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BRITISH JOURNAL OF CANCER

VOL. XV SEPTEMBER, 1961 NO. 3

THE INCIDENCE OF LUNG CANCER IN FINLAND AND NORWAY

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Received for publication June 23, 1961

THE official mortality statistics from Finland and Norway reveal a heavy excess mortality from lung cancer in Finland.

At a WHO Study Group meeting on the epidemiology of lung cancer held in Geneva in November 1959, the difference in lung cancer frequency between the two neighbouring countries attracted much attention. It was recommended that studies of the factors that might account for the difference should be undertaken (World Health Organization, 1960).

As a first step the available material on the mortality and morbidity of lung cancer has been collected as a guide for the planning of further epidemiological studies. It is the purpose of this paper to present and discuss this material.

Finland and Norway and their populations

Finland and Norway have a common border in the extreme north (Fig. 1). The two countries are situated between almost the same latitudes: Finland between 59° and 70°, and Norway between 57° and 71° north. In spite of this, the climates are rather different in the two countries. Due to warm ocean current the average temperatures are higher in Norway.

The total areas of the two countries are about the same, 130,000 and 125,000 square miles in Finland and Norway respectively. While Finland is a country of low hills and lakes, Norway is a mountainous country.

In both countries population registries are established in every municipality. Emigration and immigration are low. In 1956 the Finnish population numbered 4,270,000 and the Norwegian one 3,440,000. Thus, the population density is low, 33 and 28 inhabitants per square mile respectively. In both countries about one-third of the population live in areas administratively classified as urban. The two capitals, Helsinki and Oslo, are of the same size, counting about 450,000 inhabitants. Neither country is heavily industrialized, but while agriculture and forestry are by far the most frequent male occupations in Finland, industry is the most frequent in Norway (Table I).

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FIG. 1.

TABLE I.—*Percentage Distribution of Male Occupations in Finland and Norway. 1950 Census*

Country	Agriculture, forestry, fishing (%)	Industry, construction (%)	Other occupations (%)
Finland	46	32	22
Norway	31	40	29

The age distribution of the two populations is widely different (Fig. 2). The Finnish population is one of the youngest and the Norwegian one of the oldest in Europe. The difference in age distribution is particularly marked in males, and must be taken into consideration when health statistics for the two countries are to be compared.

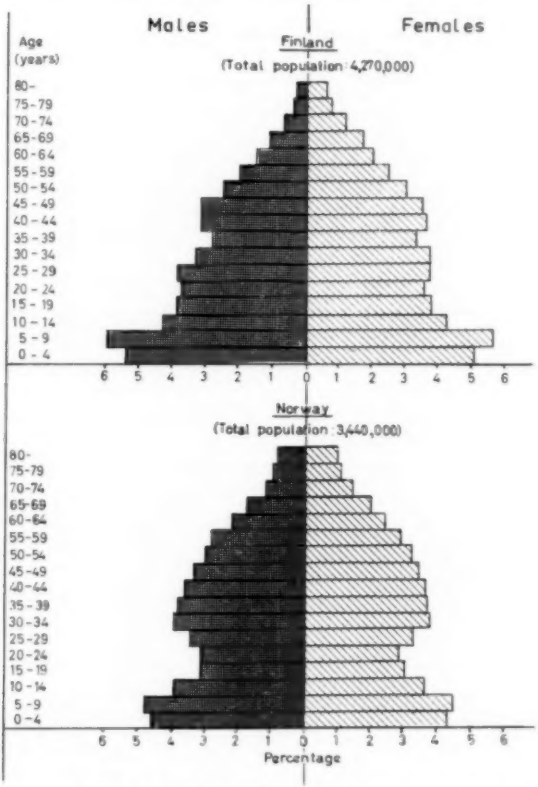

FIG. 2.—Percentage distribution of the Finnish and the Norwegian population as at January 1, 1956.

Sources of the statistical data

The data on mortality are based on the official statistics published by the Central Bureaux of Statistics of the two countries. No data are available on lung cancer mortality in Finland before 1936, when the Scandinavian List of Causes of Death of 1926 was introduced in that country. From 1951 on, the International List of 1948 has been used. In Norway the Scandinavian List was used from 1927–40. The International List of 1938 was introduced in 1941. The International List of 1948 has been used since 1951. This classification, in contrast to the preceding ones, distinguishes between "Malignant neoplasm of bronchus and trachea, and of lung specified as primary" (International List No. 162) and "Malignant neoplasm of lung, unspecified as to whether primary or secondary" (International List No. 163). Cancer of the lung in this context is thus designated by the following numbers:

List	*No.*
Scandinavian List . . .	Finland 7005, Norway 7027
Detailed International List of 1938	47 b
" " " " 1948	162 + 163

The data on morbidity are based on the material of the Cancer Registries of the two countries.

In Finland cancer registration was introduced on a voluntary basis in 1953. All hospitals, laboratories of pathology and private practitioners are urged to report all new cases of cancer that come to their attention. A compensation of 50 Finnish Marks (approximately one shilling) is paid for each report. Information on all deaths with cancer mentioned on the death certificate is submitted to the Registry. For cases reported by pathologists only or from death certificates supplementary information is requested from the hospitals or the practitioners (Saxén and Korpela, 1958).

In Norway compulsory cancer registration was introduced in 1952. The sources for collection of the material are the same as in Finland. The main difference between the reporting system of the two Registries is that cases of cancer are supposed to be reported once only to the Finnish Registry whereas the Norwegian Registry requests a report every time a patient is admitted to hospital for his malignant disease. Other differences in the reporting systems are slight (Pedersen and Magnus, 1959).

Both Registries classify the cases by site in accordance with the International Statistical Classification. The analysis of morbidity in this paper is based on Int. List No. 162 only (malignant neoplasm of bronchus and trachea, and of lung specified as primary). Salivary gland tumours of the lung have been excluded from the present material.

The detailed data on mortality and morbidity from lung cancer are given in the appendix.

Mortality

The mortality from all causes is higher in Finland than in Norway, and the difference is particularly marked among males (Fig. 3). Except for a rise during the Second World War there has been a downward trend in the mortality rate over the last 20 years. Whereas the mortality in Finland is still decreasing, the Norwegian rates are now rather stable. Actually, among males, the rate for 1957–58 is slightly higher than for 1951–55. The excess mortality in Finland is to a great extent due to arterio-sclerotic heart disease (Table II). The ratio between the mortality rates in the two countries is, however, greatest for tuberculosis, followed by lung cancer. One might question whether the high tuberculosis mortality in Finnish males could spuriously contribute to the high recorded lung cancer mortality. This would be so if deaths from tuberculosis were misdiagnosed as lung cancer. A large fraction of the tuberculosis deaths must have been misdiagnosed for the effect to be of quantitative significance. As an example, if in the present material (Table II) 10 per cent of all tuberculosis deaths among Finnish males have been erroneously recorded as lung cancer deaths, the " true " annual lung cancer mortality rate would be reduced to 6·0 from 6·7 per 10,000 and the " true " ratio between Finland and Norway reduced to 5·4 from 6·1. Thus even frequent misdiagnosing could account for only a small part of the excess Finnish mortality.

One might also question whether the deaths classified as primary lung cancers represent deaths from cancers metastasizing to the lungs more frequently in Finland than in Norway. This is not indicated by the present material as the

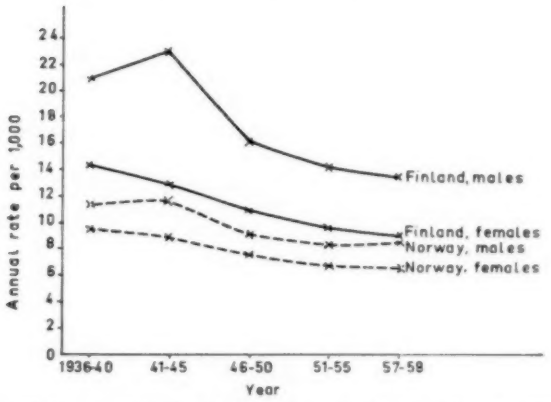

FIG. 3.—Total mortality in Finland and Norway. Annual age-adjusted rates per 100,000 (Calculated by direct standardization. Total population of Finland and Norway as at January 1, 1958, used as standard population.)

TABLE II.—*Age-adjusted Mortality Rates* from Various Causes of Death in Finland and Norway*, 1957–58

Int. List No.	Cause of death	Annual rate per 10,000					
		Males			Females		
		Finland	Norway	Ratio F : N	Finland	Norway	Ratio F : N
001–008	Respir. Tub.	6·6	0·9	7·3	2·0	0·4	5·0
162–163	Lung cancer	6·7	1·1	6·1	0·5	0·3	1·7
140–205 excl. 162–163	Other cancer	16·3	13·8	1·2	13·6	11·5	1·2
420–422	Arteriosclerotic heart disease	32·6	19·8	1·6	17·0	12·0	1·4
490–502	Pneumonia, bronchitis	5·0	4·0	1·2	4·0	4·1	1·0
All other causes		66·7	44·3	1·5	51·2	35·7	1·4
	Total	133·9	83·9	1·6	88·3	64·0	1·4

* Rates corrected with the same factors as mortality rates from all causes given in Fig. 3.

mortality in Finland from all cancers, excluding lung cancers, is higher than in Norway (Table II).

The trend in lung cancer mortality (Fig. 4) is in sharp contrast to that of the total mortality, shown in Fig. 3.

Among males the mortality is 5–6 times as high in 1957–58 as in 1934–36. The relative increase is about the same in both countries but the actual mortality in Finland is much higher than in Norway. The rate in Finland in 1936 was about the same as that in Norway in 1957–58. The trend in the recorded lung cancer mortality 1935–57 is very different for the two sexes (Fig. 4). The female mortality is lower and fairly stable except for the increase in Finland from 1940 to 1950.

The common observation that lung cancer occurs more frequently in urban than in rural areas is made also in the present material (Fig. 5*a* and 5*b*). Further-

more, the male rates in the capitals, which are the largest cities, are higher than in the provincial towns. Female rates do not show this difference between cities of different size. The rates are low and subject to large random variations and are therefore not given separately for capitals and provincial towns.

The trends of the mortality rates during the 20 years are very similar in the urban areas of the two countries, while the trends in rural areas differ widely. In Norway the increase in rural areas is slight and contributes little to the total increase in lung cancer mortality. In Finland the recorded number of lung cancer deaths in rural areas has increased from slightly more than 100 to about 600 deaths per year during the 20 years although the rural population has decreased.

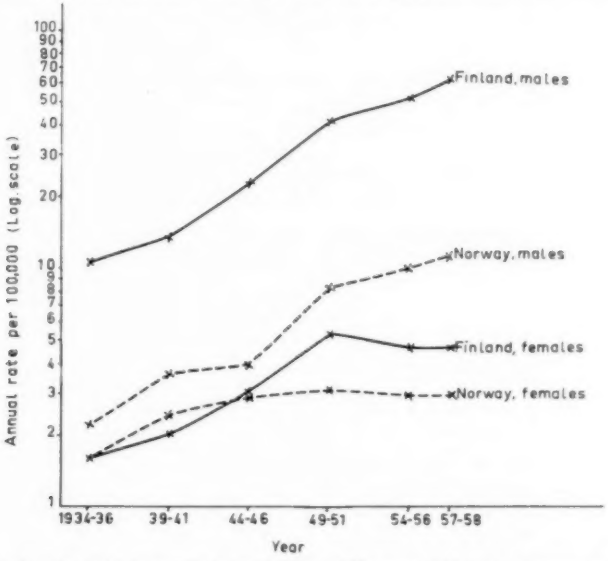

FIG. 4.—Mortality from lung cancer in Finland and Norway, 1934–58. Annual age-adjusted rates per 100,000. (Calculated by indirect standardization. Age-specific mortality rates from lung cancer combined for both sexes and the two countries used as standard rates.)

Definite conclusions should not be drawn from these data regarding the trends and present status of lung cancer mortality in the two countries. Stability over the time interval studied and uniformity between the two countries in the diagnosis, classification and reporting of lung cancer deaths is obviously lacking.

The morbidity data have the same limitations. However, as information on the individual case is collected from various sources, matching of such information provides a valuable check of the material. Furthermore the close co-operation between the Cancer Registries of Finland and Norway contributes to uniformity in the classification of the cases.

Morbidity

The data on morbidity presented below are based on the Cancer Registry materials for the years 1954–57 (Table III). Although four years is a very

short time for the study of time trends, it should be noted that the number of new cases among Finnish males increased from 782 in 1954 to 1017 in 1957.

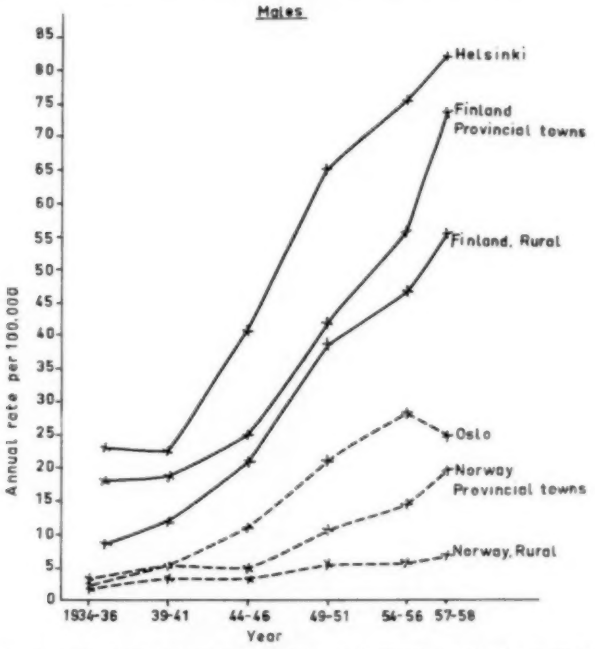

FIG. 5a.—Mortality from lung cancer in Finland and Norway, 1934–58, by residence. Annual age-adjusted rates per 100,000. (Calculated as for Fig. 4.)

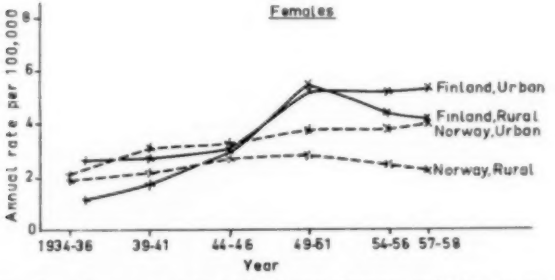

FIG. 5b.—Mortality from lung cancer in Finland and Norway, 1934–58, by residence. Annual age-adjusted rates per 100,000. (Calculated as for Fig. 4.)

Among Finnish females and in the Norwegian material no definite increase is observed. For calculation of rates the material for the four years has been combined. The rates are thus based on 3653 Finnish males, 384 Finnish females, 776 Norwegian males and 198 Norwegian females.

TABLE III.—*Incidence of Primary Lung Cancer (Int. List No. 162) in Finland and Norway 1954–57*

Residence	Number of new cases 1954	1955	1956	1957	Total 1954–57
FINLAND, MALES					
Helsinki	108	123	114	144	489
Provincial towns	226	250	287	263	1026
Rural areas	448	518	562	610	2138
The whole country	782	891	963	1017	3653
FINLAND, FEMALES					
Urban areas	46	36	43	43	168
Rural areas	54	52	52	58	216
The whole country	100	88	95	101	384
NORWAY, MALES					
Oslo	82	67	68	57	274
Provincial towns	51	52	55	63	221
Rural areas	68	59	71	83	281
The whole country	201	178	194	203	776
NORWAY, FEMALES					
Urban areas	25	27	25	36	113
Rural areas	21	25	17	22	85
The whole country	46	52	42	58	198

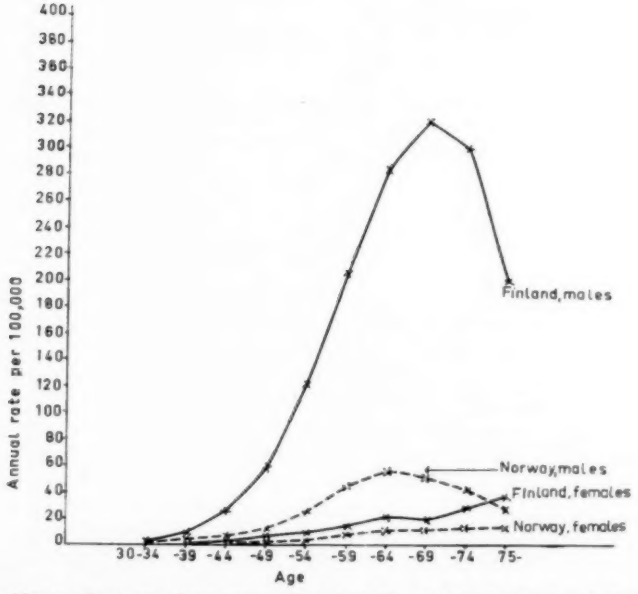

FIG. 6.—Incidence of primary lung cancer (Int. List No. 162) in Finland and Norway. Age-specific rates per 100,000. Annual average 1954–57.

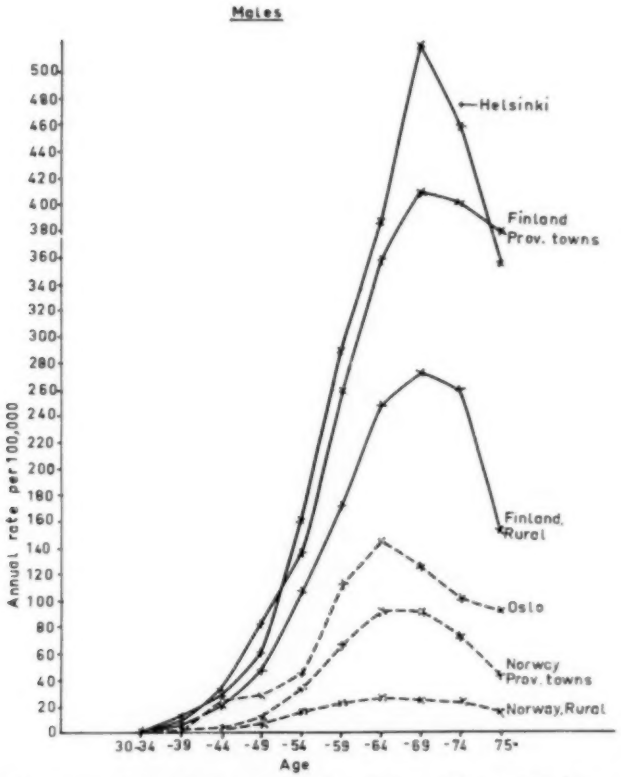

FIG. 7*a*.—Incidence of primary lung cancer (Int. List No. 162) in Finland and Norway, by residence. Age-specific rates per 100,000. Annual average 1954–57.

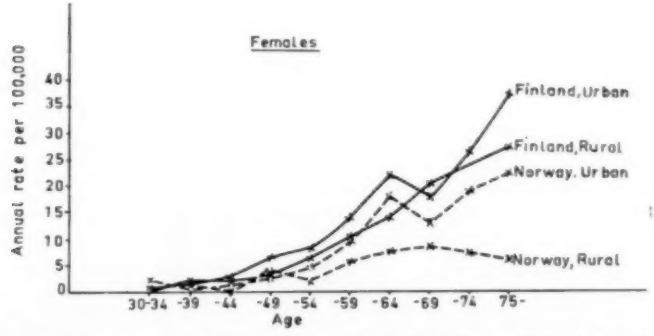

FIG. 7*b*.—Incidence of primary lung cancer (Int. List No. 162) in Finland and Norway, by residence. Age-specific rates per 100,000. Annual average 1954–57.

The age-specific rates shown in Fig. 6 clearly bring out the difference between the two countries and between the two sexes as regards the incidence of the disease. The difference is definitely more marked among males than among females. The shapes of the curves for the two sexes differ markedly. Whereas the rates for females increase up to the oldest age groups, the rates for males show a peak at about 65 years. This is a well-known observation and is considered consistent with the hypothesis of a difference in the aetiology of lung cancer in males and in females. In this context it should be pointed out that the maximum male rate is seen at a higher age in Finland than in Norway in the age groups 65–69 years and 60–64 years respectively. Random variations can hardly cause this discrepancy, but obviously it may be produced by differences

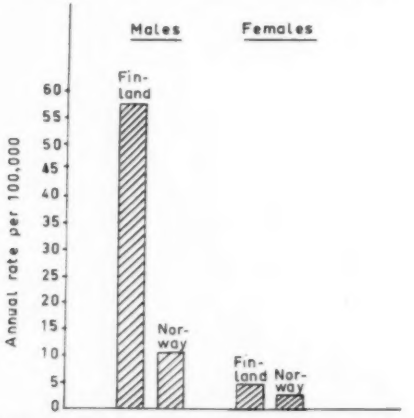

FIG. 8.—Incidence of primary lung cancer (Int. List No. 162) in Finland and Norway. Age-adjusted rates per 100,000. Annual average 1954–57. (Calculated by direct standardization. Total population of Finland and Norway as at January 1, 1956, used as standard population.)

in the diagnosis and reporting of the disease in the two countries. The age-specific rates in the capitals, provincial towns and rural areas (Fig. 7*a* and 7*b*) show very much the same picture as for the whole country. The only curve deviating from the usual pattern is that for males in rural Norway. This curve is rather flat and has no distinct peak being more similar to that for females.

To quantify the differences in incidence shown in Fig. 7–9 standardized rates have been calculated. The annual incidence rate per 100,000 among Finnish males is 57·6 and among Norwegian males 10·5. The corresponding figures for females are 4·4 and 2·3 (Fig. 8). The ratio between incidence rates in Finland and Norway is thus more than five for males and less than two for females.

For both sexes the urban/rural differences are greater in Norway than in Finland (Fig. 9). Furthermore the difference between capital and provincial towns is very slight in Finland. As a whole the ratios between incidence rates for urban/rural areas as well as capital/provincial towns are more than 50 per cent higher in Norway than in Finland. A future study of regional variations within each country may throw more light on this difference in the geographic distributions.

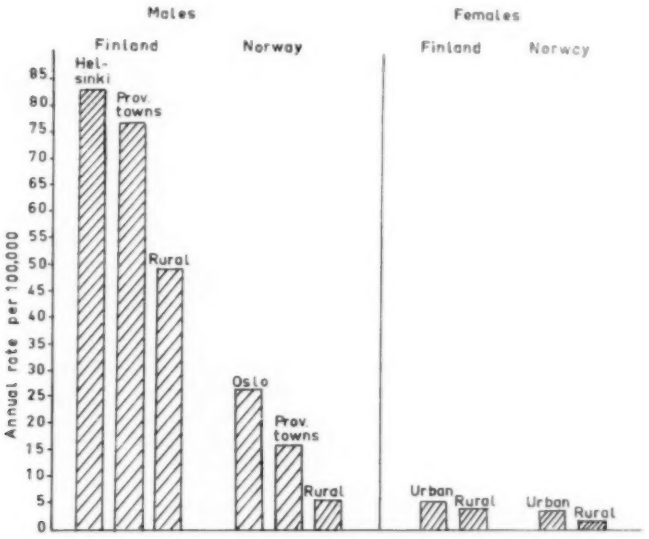

FIG. 9.—Incidence of primary lung cancer (Int. List No. 162) in Finland and Norway, by residence. Age-adjusted rates per 100,000. Annual average 1954–57. (Calculated as for Fig. 8.)

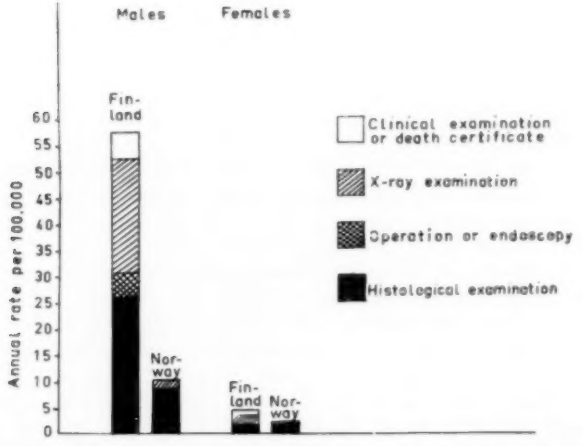

FIG. 10.—Basis of the diagnosis of new cases of lung cancer (Int. List No. 162) in Finland and Norway. Age-adjusted rates per 100,000. Annual average 1954–57. (Calculated as for Fig. 8. (Assuming that the age distribution in the four diagnosis categories is the same.)

For further evaluation of the data the cases have been classified into four groups according to the method of diagnosis (Fig. 10):

(1) Histological examination of primary tumour or metastases.
(2) Autopsy, operation or endoscopy without histological examination with or without cytological examination.
(3) X-ray examination only.
(4) Clinical examination (including cases registered on the basis of death certificate only).

Compared to Norway a greater fraction of the cases in Finland is based on clinical examination or death certificate and a smaller fraction verified histologically. However, the rate of histologically verified male cases in Finland is about 2·5 times the total male rate in Norway.

For females the contrasts are smaller. As a whole the basis of the diagnosis is poorer among females. The female cases of lung cancer are older than the male cases. As the basis of diagnosis gets poorer with increasing age, one would expect a difference in this direction. The observed difference between the sexes in the diagnosis of lung cancer, however, can only partly be explained by the difference in age.

SUMMARY AND CONCLUSIONS

Mortality and morbidity data on lung cancer are in close agreement with regard to variations within as well as between the two countries. The most striking observation in the present material is the considerable difference in the mortality and morbidity in Finnish and Norwegian males.

In the evaluation of such observations possible differences in diagnosis, classification and reporting of the disease in the two countries should be taken into account. Could the frequency of unrecognized lung cancer cases be so much higher in Norway or the frequency of cases erroneously classified as lung cancer be so much higher in Finland as to give an entirely distorted picture of the incidence of the disease in the two countries?

The great interest in lung cancer among Norwegian doctors and the good diagnostic facilities available seem to justify the assumption that the disease is not more frequently missed in Norway than in Finland. The alternative possibility that overdiagnosing might be more frequent in Finland cannot at present be excluded. A considerable fraction of " false positives " may be found among the cases not histologically verified, and almost 50 per cent of all lung cancer cases in Finland belong to this category, compared to slightly more than 10 per cent in Norway. Among the histologically verified cases one would, however, expect a negligible number only of " false positives ". This has been confirmed in a study of the histological types of lung tumours in Finland and Norway (Kreyberg and Saxén, 1961). It was established that practically all tumours represented primary lung tumours. Considering that the rate of histologically verified cases among Finnish males is more than twice the *total* rate among Norwegian males a ratio of two between incidence rates of the disease in the two countries may therefore be regarded as a minimum estimate. Even though the influence of diagnostic differences is impossible to quantify it seems reasonable to assume, however, that the ratio of about five as found in the present material gives a more correct picture.

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The observed dissimilarities in the trend and present incidence of lung cancer in Finland and Norway, as regards sex, age and geographic variation, probably reflect differences in the exposure of the Finnish and Norwegian populations to factors causally related to lung cancer. However, the aetiological aspects will not be discussed here. At present the observations made will serve solely in the planning of future studies.

REFERENCES

KREYBERG, L. AND SAXÉN, E.—(1961) *Brit. J. Cancer*, **15**, 211.
PEDERSEN, E. AND MAGNUS, K.—(1959) The Cancer Registry of Norway. Monograph No. 1.
SAXÉN, E. and KORPELA, A.—(1958) *Ann. Chir. Gyn. Fenn.*, **47**, Suppl. 79.
WORLD HEALTH ORGANIZATION—(1960) *Tech. Rep. Wld Hlth Org.*, No. 192.

APPENDIX TABLE I.—*Mortality from Cancer of the Lung in Finland and Norway*, 1934–58

FINLAND

	Year					
	36	39–41	44–46	49–51	54–56	57–58
	MALES					
Helsinki :						
Number of cases	24	78	131	245	350	262
Crude annual rate per 100,000	19·51	18·98	30·12	51·36	65·92	68·23
Standardizing factor	1·18	1·17	1·34	1·26	1·14	1·20
Age-adjusted annual rate per 100,000	23·0	22·2	40·3	64·8	75·1	81·8
Provincial towns :						
Number of cases	34	120	153	354	572	566
Crude annual rate per 100,000	14·66	15·63	18·82	27·90	38·91	53·00
Standardizing factor	1·23	1·19	1·32	1·51	1·42	1·39
Age-adjusted annual rate per 100,000	18·1	18·6	24·8	42·1	55·4	73·7
Rural areas :						
Number of cases	103	443	665	1183	1517	1213
Crude annual rate per 100,000	6·76	9·79	15·73	29·38	36·64	44·40
Standardizing factor	1·25	1·20	1·32	1·31	1·27	1·24
Age-adjusted annual rate per 100,000	8·5	11·8	20·7	38·5	46·5	55·1
The whole country :						
Number of cases	161	641	949	1782	2439	2041
Crude annual rate per 100,000	8·57	11·23	17·33	30·87	39·72	48·78
Standardizing factor	1·24	1·20	1·32	1·35	1·29	1·27
Age-adjusted annual rate per 100,000	10·7	13·4	22·8	41·7	51·2	62·0
	FEMALES					
Urban areas :						
Number of cases	11	40	49	110	127	97
Crude annual rate per 100,000	2·50	2·73	3·05	5·10	5·23	5·57
Standardizing factor	1·05	0·99	1·00	1·03	0·97	0·96
Age-adjusted annual rate per 100,000	2·6	2·7	3·0	5·3	5·1	5·3
Rural areas :						
Number of cases	17	73	120	207	178	114
Crude annual rate per 100,000	1·14	1·62	2·72	4·99	4·21	4·09
Standardizing factor	1·09	1·06	1·09	1·08	1·03	1·01
Age-adjusted annual rate per 100,000	1·2	1·7	3·0	5·4	4·3	4·1
The whole country :						
Number of cases	28	113	169	317	305	211
Crude annual rate per 100,000	1·45	1·90	2·81	5·03	4·58	4·66
Standardizing factor	1·08	1·05	1·06	1·06	1·01	0·99
Age-adjusted annual rate per 100,000	1·6	2·0	3·0	5·3	4·6	4·6

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Appendix Table I—*contd.*

Norway

	Year					
	34–36	39–41*	44–46	49–51	54–66	57–58
Males						
Oslo :						
Number of cases	8	11	44	133	212	126
Crude annual rate per 100,000	2·22	4·51	11·64	22·50	34·14	29·72
Standardizing factor	1·09	1·10	0·93	0·92	0·82	0·82
Age-adjusted annual rate per 100,000	2·4	5·0	10·8	20·7	28·0	24·4
Provincial towns :						
Number of cases	18	25	38	96	143	132
Crude annual rate per 100,000	2·36	4·79	4·49	10·92	15·28	20·75
Standardizing factor	1·36	1·03	1·01	0·96	0·92	0·92
Age-adjusted annual rate per 100,000	3·2	4·9	4·5	10·5	14·1	19·1
Rural areas :						
Number of cases	50	60	96	176	193	174
Crude annual rate per 100,000	1·61	2·78	2·79	5·18	5·38	7·14
Standardizing factor	1·21	1·14	1·06	1·01	0·96	0·92
Age-adjusted annual rate per 100,000	2·0	3·2	3·0	5·2	5·2	6·6
The whole country :						
Number of cases	76	96	178	405	548	432
Crude annual rate per 100,000	1·80	3·28	3·28	8·32	10·65	12·36
Standardizing factor	1·22	1·11	1·03	0·99	0·93	0·91
Age-adjusted annual rate per 100,000	2·2	3·6	3·9	8·2	9·9	11·2
Females						
Urban areas :						
Number of cases	27	31	55	78	87	66
Crude annual rate per 100,000	1·99	3·44	3·85	4·63	4·95	5·52
Standardizing factor	1·04	0·89	0·83	0·80	0·74	0·74
Age-adjusted annual rate per 100,000	2·1	3·1	3·2	3·7	3·7	4·1
Rural areas :						
Number of cases	40	45	94	98	94	60
Crude annual rate per 100,000	1·30	2·11	2·79	3·00	2·74	2·58
Standardizing factor	1·08	1·01	0·96	0·92	0·88	0·85
Age-adjusted annual rate per 100,000	1·4	2·1	2·7	2·8	2·4	2·2
The whole country :						
Number of cases	67	76	149	176	181	126
Crude annual rate per 100,000	1·51	2·50	3·10	3·55	3·49	3·58
Standardizing factor	1·07	0·97	0·92	0·88	0·83	0·81
Age-adjusted annual rate per 100,000	1·6	2·4	2·9	3·1	2·9	2·9

* No data available for the year 1940.

APPENDIX TABLE III.—*Annual Age-specific and Age-adjusted Rates per* 100,000 *of New Cases of Primary Lung Cancer (Int. List No.* 162), *in Finland and Norway,* 1954–57, *According to Sex, Age and Residence*

Residence	Age-group (years) 0–19	20–24	25–29	30–34	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74	75–	Total crude rate	Total age-adjusted rate
FINLAND, MALES															
Helsinki	0·4	—	—	1·9	11·5	28·4	59·8	160·6	285·6	385·6	518·9	456·6	353·3	69·5	83·6
Provincial towns	0·3	0·7	1·2	1·3	8·5	28·8	80·4	134·3	257·1	355·9	406·8	400·0	376·3	52·2	76·5
Rural areas	0·1	0·2	0·5	1·4	6·7	20·6	45·7	107·2	171·7	246·7	272·0	259·0	150·4	38·8	49·0
The whole country	0·2	0·3	0·6	1·4	7·7	23·6	56·2	119·6	203·2	283·7	319·5	299·3	197·5	43·6	57·6
FINLAND, FEMALES															
Urban areas	—	0·4	0·8	0·8	1·7	2·4	6·5	8·3	13·6	21·9	17·8	26·1	37·2	5·2	5·2
Rural areas	—	0·3	—	—	2·1	2·3	3·1	6·3	10·4	13·9	20·0	23·8	27·2	3·8	4·0
The whole country	—	0·3	0·3	0·3	1·9	2·3	4·5	7·1	11·7	17·0	19·2	24·6	30·6	4·3	4·4
NORWAY, MALES															
Oslo	—	—	—	1·5	3·0	21·0	27·9	44·4	111·4	143·5	126·5	101·9	92·3	32·9	26·7
Provincial towns	—	—	2·3	—	3·0	3·2	11·5	32·4	66·0	91·9	91·4	72·1	41·4	17·9	16·1
Rural areas	—	0·3	1·2	0·3	2·0	2·7	7·5	15·6	21·8	25·5	24·1	23·4	15·3	5·9	5·7
The whole country	—	0·2	1·3	0·4	2·3	5·2	10·7	23·2	44·0	56·0	50·4	41·5	27·5	11·3	10·5
NORWAY, FEMALES															
Urban areas	0·2	—	0·7	2·3	0·5	1·7	2·8	4·6	9·4	17·7	12·8	18·8	22·3	4·8	3·6
Rural areas	—	—	—	—	1·8	—	3·5	1·9	5·4	7·4	8·3	7·5	6·1	1·9	1·7
The whole country	0·0	—	0·2	0·8	1·3	0·6	3·2	3·0	7·0	11·4	10·0	11·8	11·8	2·9	2·3

APPENDIX TABLE II.—*Number of New Cases of Primary Lung Cancer (Int. List No. 162), in Finland and Norway, 1954–57, According to Sex, Age and Residence*

Residence	Age group (years) 0–19	20–24	25–29	30–34	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74	75–	Total
FINLAND, MALES														
Helsinki	1	—	—	1	6	16	34	71	100	101	85	45	29	489
Provincial towns	2	1	2	2	11	41	110	151	206	192	152	90	76	1026
Rural areas	3	1	2	5	20	67	152	299	392	437	374	245	141	2138
The whole country	6	2	4	8	37	124	296	521	698	730	611	380	236	3653
FINLAND, FEMALES														
Urban areas	—	1	2	2	4	6	16	17	23	29	19	20	29	168
Rural areas	—	1	—	—	7	8	11	19	28	30	37	33	42	216
The whole country	—	2	2	2	11	14	27	36	51	59	56	53	71	384
NORWAY, MALES														
Oslo	—	—	—	1	2	14	18	28	64	66	39	20	22	274
Provincial towns	—	—	2	—	3	3	10	25	46	53	40	23	16	221
Rural areas	—	1	4	1	7	9	21	42	53	51	38	28	26	281
The whole country	—	1	6	2	12	26	49	95	163	170	117	71	64	776
NORWAY, FEMALES														
Urban areas	1	—	1	4	1	3	5	8	15	23	13	15	24	113
Rural areas	—	—	—	—	6	—	10	5	13	15	14	10	12	85
The whole country	1	—	1	4	7	3	15	13	28	38	27	25	36	198

APPENDIX TABLE IV.—*Basis of the Diagnosis of the New Cases of Primary Lung Cancer (Int. List No. 162) in Finland and Norway, 1954–57, according to Sex and Residence*

Residence	Histological examination No.	%	Autopsy, operation or endoscopy No.	%	X-ray examination only No.	%	Clinical examination or death certificate No.	%	Total
FINLAND, MALES									
Helsinki	377	77·1	33	6·7	65	13·3	14	2·9	489
Provincial towns	480	46·8	89	8·7	384	37·4	73	7·1	1026
Rural areas	811	37·9	160	7·5	942	44·1	225	10·5	2138
The whole country	1668	45·7	282	7·7	1391	38·1	312	8·5	3653
FINLAND, FEMALES									
Urban areas	72	42·9	13	7·7	61	36·3	22	13·1	168
Rural areas	56	25·9	10	4·7	114	52·7	36	16·7	216
The whole country	128	33·3	23	6·0	175	45·6	58	15·1	384
NORWAY, MALES									
Oslo	248	90·5	11	4·0	14	5·1	1	0·4	274
Provincial towns	173	78·3	14	6·3	34	15·4	—	—	221
Rural areas	207	73·7	26	9·2	48	17·1	—	—	281
The whole country	628	80·9	51	6·6	96	12·4	1	0·1	776
NORWAY, FEMALES									
Urban areas	90	79·6	7	6·2	16	14·2	—	—	113
Rural areas	49	57·6	18	21·2	18	21·2	—	—	85
The whole country	139	70·2	25	12·6	34	17·2	—	—	198

THE EPIDEMIOLOGY OF SKIN CANCER IN QUEENSLAND: THE INCIDENCE

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IT has been well known for many years that the incidence of skin cancer in Australia is very high. This is more especially true of Queensland than the other Australian States, and the same probably holds for the Northern Territory, though there are no figures readily available. There is also good evidence that other tropical and sub-tropical countries have a high incidence of skin cancer amongst the white population of North European descent. Details for the Transvaal are given by Cohen *et al.* (1952); for East Africa by Piers (1948), and for the Southern States of the U.S.A. by Auerbach (1961). Social customs, economic factors and labour conditions in these countries are different from those in Australia, so that direct comparisons of statistical data should only be made with caution. In Australia there is virtually no coloured labour, and whites do manual work in all climates.

This present study has been designed to provide exact epidemiological information about skin cancer in Queensland, with a view to correlating it with the results of direct measurements of ultraviolet radiation that are currently being made by the Department of Physics in the University of Queensland. Although previous Australian authors have drawn attention to the high incidence of skin cancer (Molesworth, 1928; Belisario, 1959) it is believed that this is the first attempt to provide systematic statistics that are comparable for different parts of the State.

Geographical and Population Features

Queensland is well adapted for this epidemiological study because the population groupings fall into fairly well defined limits, and are widely spread, so that differences in response to climatic factors should be clearly apparent.

The State of Queensland covers an area of 670,000 square miles, and has a population of 1,318,259 (1954 Census). The southern border of the State lies on latitude 29° South and its northern tip, Cape York Peninsula and the Torres Strait Islands, lies between latitudes 10° and 11° South. Broadly the population can be divided into two categories. The first and major part of the population lives in the South East corner of the State, and the coastal plain from the south to as far north as Cairns (Fig. 1). The smaller section of the community lives in the arid regions to the west of the Great Dividing Range. The coastal plain has a sub-tropical to a tropical humid climate. In the south of this strip dairy farming and small crop production predominate, and in the north sugar cane

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production is the most important primary industry. On the other hand, the climate of the vast sparsely populated districts to the west of the Great Divide is described as that of low latitude steppe and desert (Blair, 1942). Sheep and cattle grazing are the main occupations, though in the North West at Mt. Isa there is a well established mining industry with a related increase in density of population. The population of Mt. Isa was 7400 in 1954, and the next largest western town was Charleville, with a population of 4500.

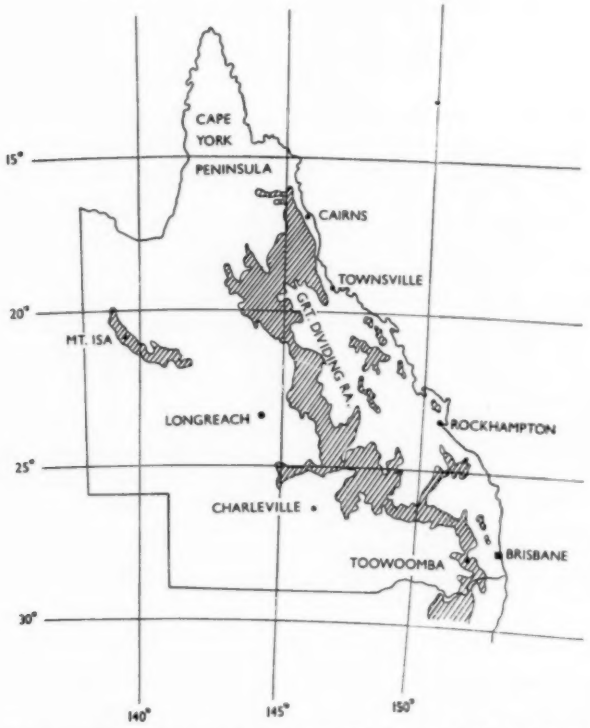

FIG. 1.—Map of Queensland, showing main geographical features and the location of the four cities surveyed.

As a result of the necessity to confine this investigation to places with reasonably large populations and with reliable hospital statistics, it is concerned only with the incidence figures for Brisbane, Rockhampton, Townsville and Cairns. It was found that in places with small populations the results were too variable, though there is a distinct clinical impression that there is a high incidence of lesions in the west. This was confirmed by a small scale postal survey carried out amongst graziers in selected western districts.

In considering cities only, bias due to exposure factors should be limited to a certain extent, though probably not entirely. In addition to this it should be

borne in mind that country dwellers are less conscientious in attending hospital than city people, which would introduce further bias if they were covered by a survey such as this rather than by " on-the-spot " sampling.

Some climatological data for the four centres covered are shown in Table I.

TABLE I.—*Climatological Factors in Survey Areas*

	Latitude in degrees S.	Population, 1954 Census	Average annual sunshine (hours)	Average rainfall	Mean daily temperature
Brisbane	27° 30′	502,320	2850	40·09	69·0°
Rockhampton	23° 28′	40,670	2925	37·36	73·2°
Townsville	19° 15′	40,471	2975	43·06	76·0°
Cairns	17° 0′	21,020	2750	86·35	76·3°

Collection of Material

The estimation of the incidence of a non-fatal, non-notifiable, relatively trivial disease with a high cure rate presents a problem that is not usually met with in compiling cancer statistics, where the death rate is a good guide to the incidence rate. However, in Queensland the treatment of skin cancers is practically uniform, in that the majority of patients attend the Queensland Radium Institute. By using the records of the Institute at the Main Centre in Brisbane and at the provincial Sub-Centres at Rockhampton, Townsville and Cairns it is possible to arrive at a good estimate of the incidence of the disease. It was ascertained from general practitioners that most of the patients are referred to the Radium Institute and, in addition, quite a number report without being referred by a doctor. Using the Institute's records, it is ensured that standards of diagnosis and record keeping are equivalent in the 4 cities concerned. The estimates should, however be regarded as minimum. Basal cell and squamous cell cancers are considered together.

A random sample of about 1000 records in each of the 4 cities was taken, of patients who presented for the first time for treatment of a skin lesion, during the 10-year period 1948–57. The total number of cases of malignant disease, and the total numbers seen at each centre were known, and from this it was possible to work out the sampling fraction, and its reciprocal, the raising factor. The factors are listed in Table V of Appendix II. The numbers of cases in the samples in each 5-year age period over the age of 20 were multiplied by the appropriate raising factor, to give the age specific numbers of cases attending for treatment.

Computation of Rates

The actual incidence rates were computed for each decennial age period from the fraction :

$$\frac{\text{Numbers of new cases in each 10-year age group}}{\text{Number of susceptible persons in corresponding group}}$$

The denominator of this fraction should be noted because the incidence rates are computed on the basis of the susceptible and not the total population a " susceptible " being a person who has not previously had a skin lesion. The incidence rates are for those with first lesions only.

The method of estimating the rates and the susceptible population is as follows :

Let

n_k = number of new cases in age group k
(obtained by multiplying the corresponding number of cards by the raising factor) ;

N_k = mean number of persons in age group k ;

$p_k = \frac{n_k}{N_k}$;

$P_k = p_1 + p_2 + \ \ldots \ + p_{k-1} + \frac{1}{2} p_k$;

$Q_k = 1 - P_k$.

P_k estimates the probability that a person whose age is that given by the mid-point of the k-th age group will have had at least one lesion by that time. It may be called the " age-specific prevalence ".

The proportion of susceptibles at this age is, therefore, Q_k.

Hence, the " age-specific annual incidence rate " for first lesions at this age is estimated by

$$r_k = \frac{1}{10} \frac{p_k}{Q_k}$$

It corresponds to the " age-specific death rate " or " force of mortality " in ordinary life-tables.

Fitting Suitable Curves

The prevalence function P and the incidence rate r are connected by a simple relationship (see Appendix I). It may therefore be a matter of mathematical convenience whether we fit the curves for r directly or fit the curves for P and deduce those for r. (Certain methods of estimating the curve may make the two methods fully equivalent, though this is not the case for the " least squares " methods used here.)

A number of different probability models of carcinogenesis have been proposed, and no doubt some of the incidence curves arising from these models would fit the data. Good use might, for instance, be made of the models proposed by Armitage and Doll (1957) or by Armitage (1959). It is doubtful, however, whether skin cancer incidence rates of the kind considered in the present survey, being, as they are, averages over different social classes and different degrees of skin pigmentation, afford suitable means of testing any given model (see, for instance, Neyman (1960), and Weibull (1951)).

Conversely, the fact that a particular probability distribution function happens to " fit " the data does not necessarily imply that the usual assumptions and models leading to that distribution are applicable to the material being studied.

The principal objects of the present survey were : (i) to show that the incidence curves varied noticeably from one district to another ; (ii) to see to what extent the factors leading to differentiation among the incidence rates were themselves age-dependent ; and (iii) to provide preliminary information necessary for the later correlation of the incidence rates with ultra-violet solar radiation in the various districts.

In deciding on questions (i) and (ii) it appeared desirable to seek prevalence curves for which, through transformations to linearity, the ordinary processes of linear regression analysis might be applied. Already, in laboratory experimental work, Blum (1955) has found the integrated normal curve to be appropriate to the production of skin cancers in albino mice by ultraviolet radiation. However, it has recently been questioned whether these observations are transferable to the situation in man. Winkelman, Baldes and Zollman (1960), who performed experiments with congenitally hairless mice, did not observe inflammatory lesions or any other skin changes before the induction of tumours. In any case, as will be seen from Fig. 2, the probit transformation of the observed values of P did not produce linearity.

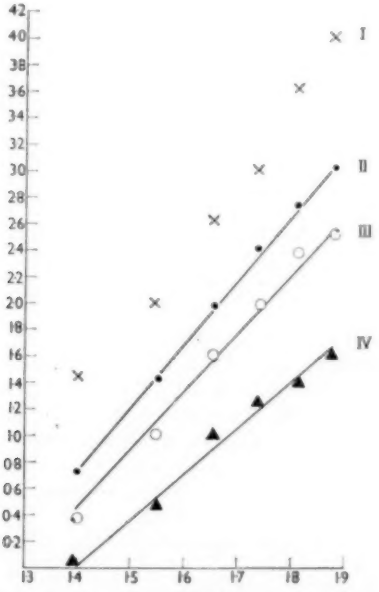

FIG. 2.—Comparison of results of various transformations seeking linearity. Townsville males.
I. Integrated normal: Y = probit $P - 1$.
II. Logistic: $3 + \log P/Q$.
III. Weibull: $3 + \log(-\log Q)$.
IV. Weibull: $3 + \log r$.

The Logistic Curve

Better results were obtained by using the logistic curve and plotting log P/Q against log (age). (In using either the integrated normal or the logistic curve it is not necessary to assume the existence of a " tolerance distribution " (Berkson, 1951).

Straight lines were fitted to the values of log P/Q, using weighted least squares. The usual weighting systems were not employed since the successive estimates of the values of P_k are not mutually independent. Weights were taken propor-

tional to the precision of the estimates (ignoring the error caused by sub-sampling from the cards). The fitted values of r were obtained from those of P by means of formula (2) of Appendix I. Chi-square tests of closeness of fit were performed by calculating the expected number of cards in each age group and comparing them with the numbers actually recorded.

Table V of Appendix II shows the observed and fitted values of r, as well as the appropriate values of chi-square. Only in one case out of the eight, namely Cairns males, was the value of chi-square large enough to indicate an apparent discrepancy from hypothesis, and this is obviously attributable to the vagaries of sampling, as can be seen from the irregularities in the sequence of observed values of r.

The equations of the 8 straight lines are given in Table VI of Appendix II.

The Weibull Curve

It has frequently been noted that certain types of age-specific death rates from carcinoma display linearity when the logarithm of the rate is plotted against the logarithm of the age. In other cases there is a marked departure from linearity, the implications of which have been discussed by Armitage and Doll (1957) and others.

In the present case linearity seemed sufficiently well marked to justify trying this system of curves. It should be noted (Appendix I) that if the r-system behaves in this way then the P-system conforms to the "Weibull distribution" (Weibull, 1951) which has many applications to life-testing data and to certain classes of biological data as well. For the Weibull P-system the graph of log ($-$ log Q) is linear when plotted against log (age).

To provide a comparison with the logistic system it was decided to fit the values of P by the Weibull distribution as well as fitting the values of log r directly. Table V of Appendix II shows the results of each process. It also gives (for comparison with the logistic system) the values of chi-square appropriate to the Weibull P-system. It will be seen that there is little to choose between this system and the logistic. If the Weibull system were accepted the logical thing to do would be to fit the values of log r directly.

Table VI of Appendix II gives the formulae for all 24 straight lines obtained by the various methods. (According to the theory of Appendix I, the slope parameters for log ($-$ log Q) should exceed those for log r by 1 when the Weibull system is used. That this does not exactly occur arises from the fact that the least squares solutions are not invariant under the transformation from P to r.)

If a set of parallel lines is fitted to all 8 districts the common slope parameter is that given in the same table.

Graphs

The following graphs are presented :

Results of fitting various curves to the data for Townsville males (Fig. 2).

Results of fitting straight lines to log P/Q versus log (age) — logistic method (Fig. 3).

Results of fitting straight lines to log r versus log (age) (Fig. 4).

Curves for r versus age obtained by fitting a set of parallel straight lines to the data for log r versus log (age) (Fig. 5).

Regression Analysis

A regression analysis was performed on each of the 3 sets of data for the purposes of: (i) testing for parallelism of the 8 curves in each set; (ii) detecting significant differences among the prevalence or incidence rates for the various

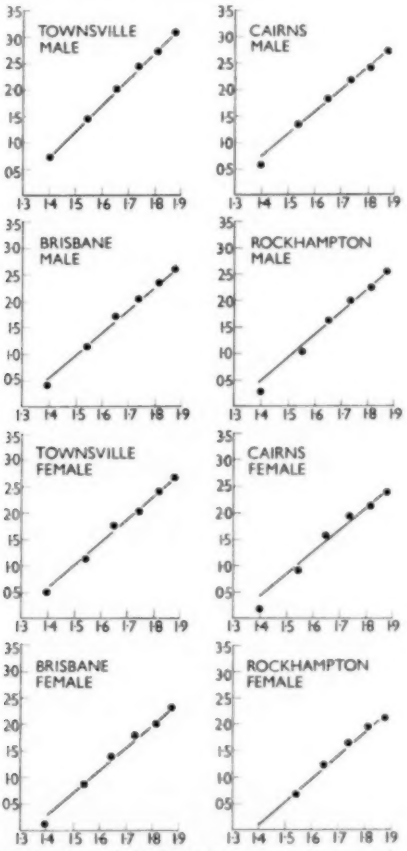

FIG. 3.—Application of the logistic curve to the prevalence rates.
Ordinates—3 + log P/Q.
Abscissae—log (age).

localities as well as differences between the sexes. Procedures appropriate to testing all contrasts in an analysis of variance were used, and the 1 per cent level of significance employed.

The somewhat lengthy numerical details of the analyses will not be presented here, but the following summary should suffice:

(i) There were no significant variations from parallelism among the 8 lines of each set under any of the 3 systems used.

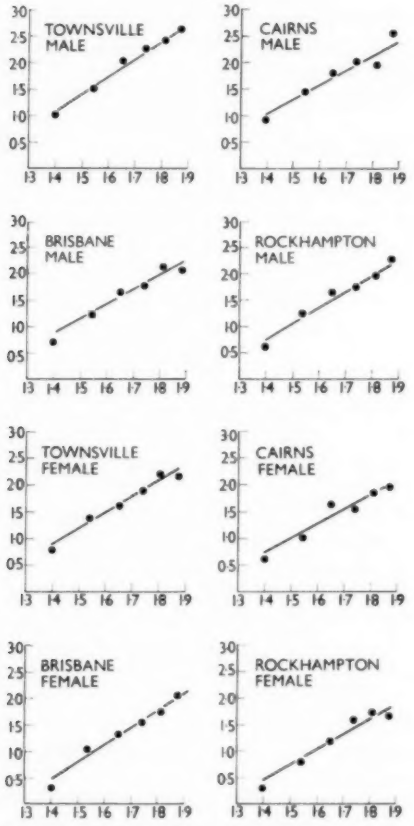

FIG. 4.—Straight lines fitted to the values of 4 + log r (ordinates) versus log (age) (abscissae)

(ii) Sex differences were of a high order of significance in all 3 cases.

(iii) For the prevalence curves, P, the following significant differences emerged (T = Townsville, C = Cairns, R = Rockhampton, B = Brisbane. The symbol " > " stands for " is significantly greater than ").

Method of fitting	Males	Females
Logistic	T > C, B and R C > R	T > C, B and R C > R B > R
Weibull	T > C, B and R C > B and R	T > C, B and R C > R B > R

It will be seen that the only conflict is that the contrast C > B for males appeared under the Weibull system but not under the logistic. In relation to the

direct fitting of straight lines to log r the only significant differences at the " 1 per cent level " were T > C, B and R for both sexes. It is to be expected, of course, that significant differences will be more easily established on the prevalence data than on the annual incidence data. Indeed, if a lower level of significance is admitted (the " 10 per cent level ") the significant differences for log r are exactly the same as for the logistic P-system given above.

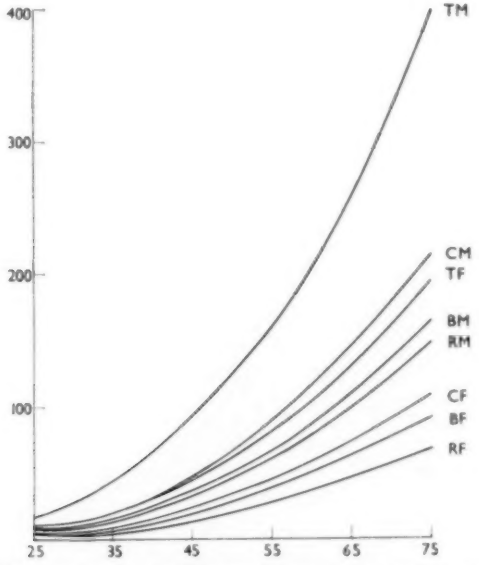

FIG. 5.—Annual incidence curves, per 10,000 population, derived by fitting parallel lines to log r versus log (age).

T—Townsville ; C—Cairns ; B—Brisbane ; R—Rockhampton.
M—Male ; F—Female.
Abscissa—Age in years.

The implications are that separate prevalence and incidence curves may be drawn for Townsville, Cairns and Rockhampton for both males and females, while there is also a likely separation between Brisbane and Rockhampton females. Some problems arising from the orderings given above are discussed later.

Estimates of Average Risk

The average annual risk of incurring a first lesion over the period 20 years to 80 years of age was estimated from the mean ordinate of the incidence curve over this range. Two sets of results are presented in Table II below, namely, estimates based on the values of r as obtained by fitting logistic curves to the prevalence data, and estimates based on the values of r obtained by fitting Weibull curves directly to the values of r.

TABLE II.—*Average Annual Risk of Incurring a First Lesion Over the Period from 20 to 80 Years of Age*

Locality	Average annual risk			
	Males		Females	
	Logistic method	Weibull method	Logistic method	Weibull method
Townsville	0·0150	0·0157	0·0074	0·0073
Cairns	0·0082	0·0080	0·0043	0·0040
Brisbane	0·0064	0·0062	0·0035	0·0035
Rockhampton	0·0056	0·0056	0·0027	0·0024

Estimates of Prevalence

Using the distributions fitted to the values of P it is possible to estimate, for for any district, the overall " prevalence " (that is, the percentage of people who, at any particular time, will be found to have at least one active or old lesion). This is best done for a population that has been standardised with respect to its age distribution and then allocated the appropriate district prevalence figure for each age group.

Table III shows such estimates for a standard population obtained by pooling the 4 district populations for the particular sex. (The assumption is, of course, that of constancy of the age-specific rates over the life-time of an individual.) Estimates are confined to that section of the population between 20 and 80 years of age. Separate estimates were obtained from the logistic and the Weibull P-distributions.

TABLE III.—*Estimated Prevalence in Age-standardised Populations in the 4 Localities among Persons between 20 and 80 Years of Age*

Locality	Estimated prevalence per cent			
	Males		Females	
	Logistic method	Weibull method	Logistic method	Weibull method
Townsville	12·84	12·77	7·70	7·67
Cairns	8·19	8·13	4·75	4·72
Brisbane	6·28	6·24	3·75	3·71
Rockhampton	5·49	5·41	2·84	2·79

Equivalent Ages

By fitting a set of parallel lines to the appropriate function of P, and employing the usual techniques of " relative potency " estimation in dosage trials one can calculate indices showing what ages in the various localities are equivalent as far as prevalence is concerned. For example the prevalence at age t years in Townsville is equivalent to the prevalence at age 1·22 t years in Brisbane, according to the logistic method, and to an age of 1·21 t years according to the Weibull method, and this is true for all values of t between 20 and 80.

Similar results were obtained, by each method, for the other centres. The agreement between the two methods and between the sets of results obtained from the data for males and the data for females is evident from the following

table (Table IV). The factor 1·22 for Townsville may be called the " relative intensity factor for ages " for Townsville as compared with Brisbane.

TABLE IV.—*Relative Intensity Factors for Age*

Locality	Relative intensity factor					
	By logistic method			By Weibull method		
	From male data	From female data	Average	From male data	From female data	Average
Brisbane	1·00	1·00	1·00	1·00	1·00	1·00
Rockhampton	0·96	0·93	0·95	0·96	0·93	0·95
Townsville	1·23	1·21	1·22	1·22	1·21	1·21
Cairns	1·08	1·06	1·07	1·07	1·06	1·06

INTERPRETATION OF RESULTS AND CONSIDERATION OF CLIMATIC FACTORS

The most noticeable feature of the curves just obtained is that their linear transforms are parallel if plotted against log (age). It appears from this survey that the cities differ only in the different incidence rates and that the factors causing the differentiation operate uniformly at all ages. The reaction patterns do not appear to differ in any way qualitatively, as is shown in another paper (Carmichael, 1961).

This observation suggests that there might be a type of dose response relationship to ultraviolet radiation. However two points need explanation :

(i) Why the Townsville rates are so much higher than those for both Cairns and Rockhampton ;
(ii) Why the Brisbane and Rockhampton rates are similar in the case of the males.

In Table I the sunshine hours recorded are shown, and it will be noticed that Cairns has less sunshine than Townsville, although it is nearer the Equator. This in itself might be enough to explain the anomaly, but population characteristics enter into the question as well. In Cairns there is a higher proportion of those Italian born or of Italian descent than in Townsville. Italians do develop skin cancer but with nothing like the same frequency as those of Anglo-Saxon or Celtic origin. Economic status probably also plays a part ; Townsville is an industrial city and is the commercial centre and port for a large area of the North West of the State. Cairns, on the other hand, is more of a tourist resort, and has little hinterland. The major industrial activity is concerened with sugar production and export.

There are several factors that influence the intensity of solar radiation received at the earth's surface :

(i) Latitude and air mass.
(ii) Solar constant (intensity of radiation outside atmosphere).
(iii) Concentration of ozone in the atmosphere, which is a very potent absorber of ultraviolet radiation.
(iv) Dust and water vapour in the atmosphere. The former absorbs and scatters ultraviolet, and the latter absorbs infrared radiation.
(v) Sky radiation as opposed to direct radiation.

Prediction of the incident ultra-volet radiation from this complex system of variables is not yet possible to a reliable degree, so it is necessary to make direct measurements. This is in progress. With these meteorological variables must be considered certain human factors. Firstly there are unmeasurable characteristics such as use of protective clothing, hats, and time of exposure to the sun. While it is generally conceded that ultraviolet radiation is responsible for this high incidence of skin cancer, it must be remembered that it is not yet possible to treat effects due to this form of radiation in the same quantitative ways that are applicable to effects due to ionising radiation.

Migratory movements of the population are also likely to affect the skin cancer incidence rate, and it is well known in Queensland that graziers tend to retire to the coastal cities. Townsville attracts the elderly population of the north west rather than Cairns, which could be a factor responsible for the high rate of skin cancer in the upper age group. In the same way, of course, a large incoming young migratory population would tend to lower the incidence rates in the earlier age groups. To collect information concerning these factors would require an extensive sample census, and the results would be of uncertain interpretation, particularly as the individual's susceptibility to solar radiation seems to alter through the years. Middle-aged and elderly people are usually a good deal more cautious about exposure to hot tropical sun than are children and young adults. In connection with exposure to the sun, it should be noted that it is quite possible to receive an erythemal dose of ultraviolet radiation in Queensland without being exposed directly to the sun, i.e. from sky radiation.

It may be that considerations such as these are responsible for the figures for the Brisbane males being similar to those for Rockhampton.

The slopes of the lines for log (incidence) against log (age) cluster about the value 3, which is in contrast to the values for death rates due to cancer, which are about 5 or 6 (Armitage and Doll, 1954). As the curves we have calculated are equivalent to death rates for lethal diseases with short survival times, this poses further problems for the multiple mutation theory of carcinogenesis proposed by Nordling (1953) and also by Stocks (1953) who used cohort death rates, unless it be assumed that neoplasms in different organs are the result of different numbers of mutations. Solar cancers might be regarded as one of the simplest and most straightforward examples of carcinogenesis that can be studied in man. However, even in this instance there is disagreement about the importance of the melanin pigment and thickness of the stratum corneum as protective factors against solar radiation (Mackie and McGovern, 1958; Blum, 1959; Thomson, 1955). Until some of the biological difficulties are resolved, the appropriateness of various theoretical models of carcinogenesis cannot be adequately assessed.

SUMMARY

The age specific incidence rates of skin cancer in four coastal cities in Queensland are calculated and discussed. On the assumption that ultraviolet radiation is the causative factor, it is suggested that the only difference in response to the stimulus by the four populations is one of frequency of incidence. This arises from the observation that the curves are parallel for each of the four cities. Several methods of fitting lines to the data are described.

This investigation was carried out while one of us held a Medical Research Fellowship at the University of Queensland. We are indebted to Dr. A. G. S. Cooper, Director of the Queensland Radium Institute, who allowed free use of the records of the Institute, and who helped with the numerous problems that arose during the course of the survey.

REFERENCES

ARMITAGE, P.—(1959) *J. nat. Cancer Inst.*, **23**, 1313.
Idem AND DOLL, R.—(1954) *Brit. J. Cancer*, **8**, 1.—(1957) *Ibid.*, **11**, 161.
AUERBACH, H.—(1961) *Publ. Health Rep.*, **76**, 345
BELISARIO, J. C.—(1959) ' Cancer of the Skin '. London (Butterworth).
BERKSON, J.—(1951) *Biometrics*, **7**, 327.
BLAIR, T. A.—(1942) ' Climatology '. New York (Prentice-Hall).
BLUM, H. F.—(1955) in ' Radiation Biology,' ed. A. Hollaender. New York (McGraw-Hill).—(1959) ' Carcinogenesis by Ultraviolet Light '. Princeton University Press.
CARMICHAEL, G. G.—(1961) *Brit. J. Cancer*, **15**, 425.
COHEN, L., SHAPIRO, M. P., KEEN, P. AND HENNING, A. J. H.—(1952) *S. Afr. med. J.*, **26**, 932.
MACKIE, B. S. AND MCGOVERN, V. J.—(1958) *Arch. Derm. Syph., N.Y.*, **78**, 218.
MOLESWORTH, E. H.—(1928) *Med. J. Aust.*, **1**, 878.
NEYMAN, J.—(1960) *J. Amer. statist. Ass.*, **54**, 625.
NORDLING, C. O.—(1953) *Brit. J. Cancer*, **7**, 68.
PIERS, F.—(1948) *Brit. J. Derm.*, **60**, 319.
STOCKS, P.—(1953) *Brit. J. Cancer*, **7**, 407.
THOMSON, M. L.—(1955) *J. Physiol.*, **127**, 236.
WEIBULL, W.—(1951) *J. appl. Mech.*, **18**, 293.
WINKELMAN, R. K., BALDES, E. J. AND ZOLLMAN, P. E.—(1960) *J. invest. Derm.*, **34**, 131.

APPENDIX I

Prevalence and incidence curves

If $P(t)$ is the probability that a lesion will occur before time t, the probability that a person who has not had a lesion before time t till get one in the interval $(t, t + \Delta t)$ is

$$r(t)\ \Delta t = \frac{p(t)\ \Delta t}{1 - P(t)}, \qquad . \quad . \quad . \quad . \quad (1)$$

so that

$$r(t) = -\frac{d}{dt} \ln \{1 - P(t)\} \qquad . \quad . \quad . \quad . \quad (2)$$

If Δt is taken as 1 year, $r(t)$ is the annual incidence rate.

Logistic curve

The 2-parameter logistic curve on $x = \ln t$ is

$$P = \frac{1}{1 + e^{-(A + Bx)}} = \frac{1}{1 + ct^{-b}}, \text{ say} \qquad . \quad . \quad . \quad (3)$$

We have

$$\log \frac{P}{Q} = a + bx, \text{ say} \qquad . \quad . \quad . \quad . \quad (4)$$

and

$$r(t) = \frac{bP}{t}, \text{ from (2).}$$

Weibull curve

The Weibull distribution is

$$P = 1 - \exp(-Ct^D) \quad . \quad . \quad . \quad . \quad (5)$$

so that

$$\ln(-\ln Q) = \ln C + Dx \quad . \quad . \quad . \quad . \quad (6)$$

and

$$\ln r = \ln(CD) + (D-1)x \quad . \quad . \quad . \quad . \quad (7)$$

Parallel lines for $\log(-\log Q)$ imply parallel lines for $\log r$, and *vice versa*.

APPENDIX II

Table V shows the observed values of r and those obtained by the use of: (i) a separate logistic curve for the prevalence data for each sex and each district; (ii) a separate Weibull prevalence curve for the same prevalence data; and (iii) a separate Weibull incidence curve obtained for each set of incidence rates by fitting the values of $\log r$ directly.

TABLE V.—*Observed Incidence Rates* (r) *per* 10,000 *Susceptibles; the Fitted Rates* $\hat{r}_l$ *Obtained from the Logistic Prevalence Curve; the Fitted Rates* $\hat{r}_w$ *Obtained from Weibull Prevalence Curves; the Fitted Rates* $\hat{r}_w'$ *Obtained by Fitting Log r Directly*

BRISBANE MALES (Raising factor = 34)

Age	No. in sample	No. in population	r	$\hat{r}_l$	$\hat{r}_w$	$\hat{r}_w'$
25	5	33,045	5	6	6	7
35	18	37,639	17	17	17	19
45	41	32,585	45	39	37	38
55	39	24,658	60	72	70	67
65	60	18,717	133	114	120	107
75	21	8,379	118	160	188	161

Chi-square*: Logistic 4·65; Weibull 6·77

ROCKHAMPTON MALES (Raising factor = 2·6)

25	4	2,675	4	5	5	5
35	16	2,702	15	15	14	15
45	38	2,509	41	34	32	32
55	38	1,978	54	62	61	59
65	44	1,450	91	100	105	99
75	35	731	163	142	167	152

Chi-square*: Logistic 4·23; Weibull 4·81

TOWNSVILLE MALES (Raising factor = 4·9)

25	7	3,156	11	10	12	12
35	19	3,136	30	37	36	37
45	49	2,615	101	92	84	85
55	59	2,109	172	176	166	163
65	53	1,559	258	269	294	282
75	21	536	412	344	477	450

Chi-square*: Logistic 1·57; Weibull 0·28

TABLE V (*Continued*)

CAIRNS MALES (Raising factor = 2·4)

25	5	1,570	8	8	9	10
35	19	1,739	27	25	24	25
45	31	1,385	57	53	50	49
55	37	1,118	91	94	91	86
65	20	714	85	143	150	138
75	25	281	329	192	228	205

Chi-square*: Logistic 14·90; Weibull 13·39

* See text.

BRISBANE FEMALES (Raising factor = 35)

Age	No. in sample	No. in population	r	r_l	$\dot{r}_w$	w
25	2	35,668	2	3	3	3
35	12	38,430	11	9	9	9
45	19	33,010	21	20	20	19
55	27	27,525	36	38	37	37
65	31	22,473	53	63	64	62
75	31	11,381	114	92	102	97

Chi-square*: Logistic 2·96; Weibull 2·34

ROCKHAMPTON FEMALES (Raising factor = 2·6)

25	2	3,012	2	2	2	3
35	7	2,857	6	7	6	7
45	15	2,633	15	15	15	15
55	28	2,110	36	29	28	26
65	29	1,750	46	49	49	42
75	14	892	45	74	78	62

Chi-square*: Logistic 4·77; Weibull 4·21

TOWNSVILLE FEMALES (Raising factor = 4·9)

25	4	3,052	6	6	7	8
35	13	3,020	23	20	20	21
45	20	2,400	43	45	43	44
55	28	1 970	78	83	81	79
65	36	1,464	151	132	136	129
75	14	666	150	182	213	195

Chi-square*: Logistic 1·13; Weibull 2·77

CAIRNS FEMALES (Raising factor = 2·4)

25	2	1,613	3	4	4	5
35	7	1,608	10	12	12	13
45	22	1,263	43	26	25	25
55	13	987	34	48	47	43
65	17	662	70	77	78	67
75	8	271	87	109	120	98

Chi-square*: Logistic 6·69; Weibull 8·84

* See text.

Table VI gives the equations of the various sets of straight lines fitted to the data.

TABLE VI.—*Straight Lines Fitted to Various Transformations of the Data: A_i ($i = 1, 2, 3$) and B_i are the Location and Slope Parameters, Respectively. [$x = log$ (age).]*

	Method of Fitting					
	Logistic to prevalence data $\log \frac{P}{Q} = A_1 + B_1 x$		Weibull to prevalence data $\log(-\log Q) = A_2 + B_2 x$		Weibull to incidence rates $\log r = A_3 + B_3 x$	
Data fitted	A_1	B_1	A_2	B_2	A_3	B_3
Brisbane males	−8·80	4·31	−8·63	4·16	−7·11	2·83
Rockhampton males	−5·56	4·31	−8·84	4·24	−7·49	3·03
Townsville males	−9·08	4·87	−8·67	4·39	−7·47	3·26
Cairns males	−8·20	4·22	−8·17	3·97	−6·90	2·78
Brisbane females	−8·70	4·26	−9·01	4·21	−7·93	3·16
Rockhampton females	−9·00	4·36	−9·28	4·30	−7·44	2·80
Townsville females	−8·53	4·37	−8·81	4·13	−7·20	2·93
Cairns females	−8·40	4·16	−8·57	4·03	−6·98	2·65
99% Confidence interval for common slope parameter		4·36 ± 0·15		4·17 ± 0·17		2·92 ± 0·38

THE EPIDEMIOLOGY OF SKIN CANCER IN QUEENSLAND: THE SIGNIFICANCE OF PREMALIGNANT CONDITIONS

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In a previous paper (Carmichael and Silverstone, 1961) a method of calculating the incidence rates of skin cancer in four Queensland coastal cities, based on hospital records was described. In this paper these same records are analysed further with respect to type and site of lesions exhibited by the patients. Only the records of patients giving addresses in Brisbane, Rockhampton, Townsville and Cairns are used, so that uniformity of some of the variables is ensured.

The concept of premalignancy is, in the main, a clinical and pathological one, though it gets some assistance from statistics in a few instances.

The statistical approach to premalignancy is essentially one of testing the association of attributes. If the proportion of people suffering from a pathological condition X in a general population is less than the proportion in a population also suffering from a cancer Y, then X and Y are positively associated, and X could be related to the genesis of the cancer. The conclusions drawn from this type of argument would be influenced by such considerations as the relative frequency of the two conditions, the prognosis of the cancer, and whether the presence of the precancerous lesion merits treatment to prevent the malignancy supervening.

It is usual to consider hyperkeratosis as premalignant for both squamous and basal cell cancer (Belisario, 1959; Payling Wright, 1958), though hyperkeratosis is more nearly related to squamous cell cancer histologically. Hyperkeratosis, or solar keratosis as it is often called, may also remain a benign lesion.

In Queensland both skin cancer and solar keratosis are very common, and it is of interest to test the association of these attributes in the samples of records already referred to.

ANALYSIS OF THE DATA

Samples of records of patients presenting at the clinics of the Queensland Radium Institute at Brisbane, Rockhampton, Townsville and Cairns, in the ten year period 1948–1957 were classified according to whether treatment had been for squamous or basal cell carcinoma or hyperkeratosis.

The following symbols were used:—

A, α: Presence or absence of basal cell cancer.
B, β: Presence or absence of squamous cell cancer.
C, γ: Presence or absence of solar keratosis.

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There are eight possible combinations of these attributes, but only seven appear in this analysis because there is no $\alpha\beta\gamma$ population. It was possible to show that there was no difference in the proportions of the lesions developed between the four cities, though there was a difference between the sexes. The frequencies are therefore amalgamated for each city and are shown in Table I. In most cases

TABLE I.—*Classification of Patients According to Types of lesions developed. Amalgamated Data from the Four Centres. Figures in brackets are percentages*

	Male	Female	Total
$A\beta\gamma$	324 (27·9)	194 (21·5)	518 (25·1)
$\alpha B\gamma$	96 (8·3)	38 (4·2)	134 (6·5)
$\alpha\beta C$	413 (35·5)	465 (51·4)	878 (42·5)
$AB\gamma$	21 (1·8)	7 (0·8)	28 (1·4)
$A\beta C$	206 (17·7)	151 (16·7)	357 (17·3)
αBC	43 (3·7)	33 (3·7)	76 (3·7)
ABC	60 (5·2)	16 (1·8)	76 (3·7)
Total	1163	904	2067

the diagnosis is a clinical one, only atypical lesions being examined histologically. The solar keratosis is a lesion that may vary from a thin keratinous plaque to a thickened hyperkeratotic area that may measure up to a centimetre in diameter.

If the frequencies in each classification are tabulated, certain inequalities are apparent. The direction of the inequalities is the same for the males as for the females, though the numbers are different. Brackets indicate frequencies.

$$(A\beta\gamma) > (A\beta C)$$
$$(\alpha B\gamma) > (\alpha BC)$$
$$(A\beta\gamma) + (A\beta C) > (AB\gamma) + (ABC)$$
$$(\alpha B\gamma) + (\alpha BC) > (AB\gamma) + (ABC)$$
$$(AB\gamma) < (ABC)$$

Any *a priori* hypothesis of premalignancy must be arbitrary, and cannot be tested statistically with any meaning. However, it is reasonable to test for independence, and to take note of divergences from expectation. Three two-way tables are relevant for the associations between the two forms of cancer and hyperkeratosis, and these are set out in Tables II, III and IV. The expected values on the basis of independence are given in brackets.

TABLE II.—*The Association Between Basal Cell Cancer and Solar Keratosis*

	Male				Female		
	A	α	Total		A	α	Total
C	266 (379·3)	456 (342·7)	722	C	167 (270·7)	498 (394·3)	665
γ	345 (231·7)	96 (209·3)	441	γ	201 (97·3)	38 (141·7)	239
Total	611	552	1163	Total	368	536	904

Except for the association between the skin cancers occurring together in the presence of hyperkeratosis in the case of females (Table IV), there is marked divergence from expectation on the hypothesis of independence. The χ^2 values

TABLE III.—*The Association Between Squamous Cell Cancer and Solar Keratosis*

	Male				Female		
	B	β	Total		B	β	Total
C	103 (136·6)	619 (584·4)	722	C	49 (69·1)	616 (595·9)	665
γ	117 (83·4)	324 (357·6)	441	γ	45 (24·9)	194 (214·1)	239
Total	220	943	1163	Total	94	810	904

TABLE IV.—*The Association Between Basal and Squamous Cell Cancer in the presence of Keratosis*

	Male				Female		
	AC	αC	Total		AC	αC	Total
BC	60 (37·9)	43 (65·1)	103	BC	16 (12·3)	33 (36·7)	49
βC	206 (228·1)	413 (390·9)	619	βC	151 (154·7)	465 (461·3)	616
Total	266	456	722	Total	167	498	665

are well outside the limits expected by chance, except for the case of the females mentioned, which has a value of 1·6042 ($0{\cdot}3 > P > 0{\cdot}2$). Between each type of skin cancer occurring separately and hyperkeratosis there is dissociation. This is not unexpected on clinical grounds for basal cell cancer, but it was anticipated that there would be a positive association between squamous cell cancer and hyperkeratosis. The patients represented in Table IV include those who are acutely sensitive to the action of ultraviolet radiation, suffer from severe solar dermatitis and develop multiple lesions.

In interpreting these findings three possibilities arise that may explain them :

(i) the patients tend only to present with and be treated for skin cancer, hyperkeratosis being largely neglected ;
(ii) treatment of hyperkeratosis prevents the subsequent development of skin cancer, and
(iii) the period of observation was not long enough.

Regarding the first possibility it will be noted that 35·5 per cent of the males and 51·4 per cent of the females are in the series for hyperkeratosis alone. The notes are well kept, and treatment for hyperkeratosis is often with superficial X-ray, which necessitates the lesions being accurately described. Concerning (ii) it should be borne in mind that solar keratoses are frequently multiple and that if a positive association exists between squamous cell cancer and keratosis the cancer patients should also show the premalignant lesion. About the third possibility, the mean time of observation was five years, which should give time for multiple lesions to declare themselves and some to develop into malignancy. It would appear that the data and statistics presented are an accurate description of the patients presenting for treatment.

The anatomical distribution of the lesions also throws light on the question. In Table V are set out the distribution of the lesions in those patients who have had only one type of lesion. It will be seen that the two cancers tend to have their own sites of predilection, and that keratosis occurs with roughly equal

TABLE V.—*The Anatomical Distribution of Lesions in Selected Sites, in Patients who have been Treated for only One Type of Lesion. Figures in brackets are percentages*

	B.C.C.	S.C.C.	Keratosis
MALE			
Forehead and temple	58 (12·1)	5 (4·8)	83 (11·2)
Periorbital region	59 (12·3)	5 (4·8)	28 (3·8)
Malar region	111 (23·1)	6 (5·7)	122 (16·5)
Nose	88 (18·3)	10 (9·5)	130 (17·6)
Ear, anterior surface	20 (4·2)	11 (10·5)	52 (7·0)
Neck	35 (7·3)	7 (6·7)	23 (3·1)
Forearm	14 (2·9)	16 (15·2)	81 (11·0)
Hand	6 (1·2)	18 (17·1)	125 (16·9)
Total	391 (81·4)	78 (74·3)	644 (87·1)
FEMALE			
Forehead and temple	38 (14·8)	5 (12·5)	72 (9·1)
Periorbital region	24 (9·3)	2 (5·0)	32 (4·0)
Malar region	43 (16·7)	8 (20·0)	172 (21·7)
Nose	67 (26·1)	4 (10·0)	197 (24·8)
Ear, anterior surface	4 (1·5)	1 (2·5)	5 (0·6)
Neck	15 (5·8)	3 (7·5)	15 (1·9)
Forearm	7 (2·7)	5 (12·5)	80 (10·1)
Hand	3 (1·2)	8 (20·0)	143 (18·0)
Total	201 (78·1)	36 (90·0)	716 (90·2)

frequency in areas of predilection of both cancers. There are some exceptions to this. For instance, in the neck the cancers are relatively frequent, but only a small proportion of the keratoses occur in this site. The suggestion is that the malignancies do not evolve through a premalignant stage.

SUMMARY AND CONCLUSIONS

Data are presented for four cities in Queensland that show that there is a tendency for both basal and squamous cell cancer to occur in the absence of hyperkeratosis. The converse also holds. The anatomical distribution of the keratoses does not follow that of one particular form of cancer, but roughly that of both, although basal cell cancers and hyperkeratoses are not likely to be confused clinically. It seems that solar keratosis is not an important premalignant lesion, but rather that it occurs independently.

In this, as in the preceding paper on the incidence of skin cancer, it must be stressed that geographical factors are specially important, and that what is true for Queensland is not necessarily true for other regions further from the Equator.

This work was carried out during the tenure of a Medical Research Fellowship at the University of Queensland. I would like to acknowledge the advice and help of Dr. A. G. S. Cooper, Director of the Queensland Radium Institute.

REFERENCES

BALISARIO, J. C.—(1959) 'Cancer of the Skin'. London (Butterworth).

CARMICHAEL, G. G. AND SILVERSTONE, H.—(1961) *Brit. J. Cancer*, **15**, 409.

PAYLING WRIGHT, G.—(1958) 'An Introduction to Pathology,' 3rd edition. London Longmans).

THE HISTOPATHOLOGY OF LUNG CANCER IN LIVERPOOL: A SURVEY OF BRONCHIAL BIOPSY HISTOLOGY

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THIS paper and the following one are the outcome of an investigation into the accuracy of diagnosis and cell typing of lung neoplasms reported in bronchial biopsy specimens. As the material and interest grew, the scope of the study was enlarged, to attempt a comparison of some of the biological features of the different cell types of lung cancer, and also to contrast the incidence of different histological types of carcinoma found in this series with their frequencies in operation specimens and post mortem specimens examined in the same period, and in the carcinomas found during the Mass X-Ray Campaign held in Liverpool in 1959.

The results of part of this investigation are presented in this paper as a brief analysis of bronchial biopsy histology based upon the experience of the ten year period surveyed.

TABLE I.—*Material of the Survey*

Diagnostic bronchoscopies in 1950–59 period	7879	
Biopsy specimens taken at bronchoscopies	2310	
Histology:		
Neoplasms diagnosed	1408	62 per cent
Benign tumours	40	
Malignant tumours	1368	
"Probable" carcinoma	48	2·1 per cent
"Possible" carcinoma	26	1·1 per cent

Material of the Survey (Table I)

The slides reviewed consist of all the bronchial biopsy specimens received in the Department of Pathology, Broadgreen Hospital, Liverpool, from the Regional Thoracic Surgical Centre at the same hospital in the period 1950 to 1959 inclusive, when 7879 diagnostic bronchoscopies were performed with 2310 specimens sent for histological diagnosis. In most cases the histological examinations were to assist the diagnosis or exclusion of lung carcinoma, but some specimens came from patients known to be suffering from some other disease, usually tuberculosis, bronchiectasis, or oesophageal carcinoma.

Four pathologists took part in reporting the sections, but over 90 per cent were reported by myself or by my colleague Dr. J. A. Campbell. From 1951 all the pathologists concerned have agreed about the nomenclature to be used and the criteria for assessing histological types of lung carcinoma.

UMI

Methods of Review

Three approaches were used, and in the following order :—

(1) Without knowing the original diagnosis made I have re-examined and re-reported all the original slides of the 1445 cases from the period 1950 to 1955 inclusive.

(2) The second part of the review consisted of a comparison of biopsy histology reports with operation specimen histology reports, in 344 cases.

In about 20 per cent of cases where the biopsy was positive a lung or lobe was later examined in the same laboratory. The same pathologists made both reports, but were usually unaware of the biopsy reports when the operation specimens were examined. This arose because all specimens for histology are numbered and entered in a book as they arrive in the laboratory, and the Chest Centre specimens represent only a fifth of the tissues examined histologically. The lack of co-ordination was appreciated early on, but it was fostered until the end of the survey period to make the subsequent comparison of biopsy and operation tissue specimen histology reports of greater value.

(3) Hospital case records, and all the records of the Liverpool Cancer Control Organisation related to the lung cancer cases notified to them in the period 1950 to 1958 inclusive have been examined. As the L.C.C.O. records provide a complete follow-up of all notified malignant disease diagnosed in the Liverpool, North Wales and Isle of Man area, including all the lung carcinomas diagnosed in the Thoracic Surgical Centre, it has been possible to trace nearly all cases in this series diagnosed histologically as lung carcinoma, and to search for later information about all patients who had bronchial biopsies, particularly those diagnosed as " probable " or " possible " carcinoma.

Histological Methods and Safeguards for the Identity of Specimens

The biopsy tissue is removed from the bronchoscopy forceps into formol sublimate fixative, the container being immediately labelled and the request form completed. Paraffin blocks are prepared, using an automatic processer, and about eight step sections are mounted on two slides and stained with haematoxylin and eosin. Sometimes sections were stained with Mayer's mucicarmine.

On the whole the histological diagnosis of lung cancer is straightforward and the grave risk of a false positive report is less likely to be made through faulty histological interpretation than from a mixing of tissues, either due to wrong labelling of specimens or mixing of tissues during processing. The dangers of careless labelling are obvious, but the risk of mixing tissues during processing has become much greater since the introduction recently of machines which process up to fifty blocks of tissue automatically from fixative to wax.

In a processer the blocks, which may be tissues from post mortem, operation, or biopsy specimens, are put into individual fine-mesh cages within a basket, which is in constant motion during the hours of processing in different reagents. Sections prepared from the spun deposit in the reagent containers show that fine particles of tissue, particularly necrotic tumours, become detached from their blocks and escape from the cages during processing and become suspended in the wax baths (Fig. 1 and 2). These particles are large enough to settle in biopsy tissue and could give rise to false diagnoses of malignancy.

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As a safeguard against this hazard, all biopsy tissues are wrapped in thin linen, and all other blocks thought to contain tumours are wrapped in gauze before going into the processer (Fig. 3), and they are only removed from their covering after leaving the processer and before final embedding in new wax.

Another place where tissues get fragmented and sometimes mixed is the warm water bath used for floating sections before mounting on slides. The water bath should be washed and refilled with fresh warm water before mounting the sections from each biopsy. This safeguard is easily carried out if the old-fashioned pudding-basin is used for floating the sections, but is impracticable when modern thermostatically-controlled baths are used.

Histological Classification of Lung Cancer

Any discussion of the frequency of different cell types of lung cancer must be preceded by definition of the nomenclature used, which is widely variable not only between different countries but between hospitals in the same city.

If a classification includes a large number of histological types, each of these becomes less sharply definable, giving more opportunity for variations in interpretation by different pathologists, and even by the same pathologist examining a slide on different occasions. For this reason the classification used has been kept as simple as possible.

It is thought that a lung cancer can differentiate in only two ways, either as a squamous carcinoma or an adenocarcinoma. All other carcinomas are undifferentiated, but I regard the oat-cell carcinoma as a specific form of undifferentiated carcinoma.

Squamous carcinoma

This diagnosis is made only when keratinisation or inter-cellular bridges between prickle cells are present. Epithelial pearls without keratinisation are not considered sufficiently specific to justify the diagnosis of squamous carcinoma, which has also to be differentiated from squamatisation over the bronchial surface of oat- and adeno-carcinomas.

The bronchial epithelium in the vicinity of squamous carcinoma often shows Bowen's disease, or intra-epidermal carcinoma. This condition has never been seen in the bronchus without there being an associated carcinoma, and in biopsy tissues a diagnosis of Bowen's disease has always implied the presence of squamous carcinoma.

Many tumours have a " squamoid " or transitional carcinoma appearance, but as they lack the specific features of squamous carcinoma they are classified as carcinoma simplex.

Adenocarcinoma

Adenocarcinoma is rarely found in biopsy specimens. To be included in this group a tumour must show some glandular or tubular structure (other than of oat-cell type), or it must be possible to demonstrate mucin secretion by the tumour cells.

As in the cases recorded by Raeburn and Walter (1956), several tumours which would otherwise have been called carcinoma simplex were classified as adenocarcinoma when special strains showed mucin in the cytoplasm of the tumour cells.

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Adenomatosis (or alveolar cell, or bronchiolar carcinoma)

This peripheral carcinoma is rarer than adenocarcinoma and the relationship between these two tumours is uncertain, though they usually have distinctive appearances. They have been well described by Hutchison (1952) and Evans (1956) and show a characteristic well differentiated columnar or cuboidal epithelium lining alveolar walls, with papillary processes occupying the alveolar spaces, and they appear to have a multifocal origin from bronchioles.

These tumours are rarely found in biopsy material; only in advanced cases when the tumour extends into the bronchial lymphatics, or when the biopsy forceps penetrate the whole bronchial wall into surrounding alveolar tissue.

Oat-cell carcinoma

This tumour is characterised by regular oval or oat-shaped nuclei in pale cytoplasm which is usually faintly eosinophilic. Cell membranes are rarely seen so the nuclei appear to be in a syncytium. Though called an oat-cell tumour it is really only an oat-nucleus tumour. The nucleus is usually small, with a sharply etched nuclear membrane, and it contains dust-like chromatin granules. Sometimes these granules are larger and fewer, but nucleoli are rarely seen. Nearly all these tumours, even in biopsy specimens, show areas where the oat-cells are "streaming", a condition where the nuclei are swollen, elongated, hyperchromatic and arranged in clumps, pallisades or whirls. This "streaming" is found most markedly in areas of tumour pinched by biopsy forceps, and oat-cells, like lymphocytes, appear to be more fragile than other cells.

In some cases, areas of tumour show nuclei arranged in tubular or rosette pattern, where the cytoplasm is paler and the cell membranes are more clearly delineated.

Many authors call these tumours anaplastic, undifferentiated, or small cell carcinomas. The term "oat-cell" has been retained here as it is considered to be a specific histological type, not to be confused with other undifferentiated carcinomas, and because though most oat-cell tumours have small nuclei about 5 per cent have nuclei as large as other carcinomas. It is the structure of the nucleus rather than its size which is considered diagnostic.

Carcinoma simplex

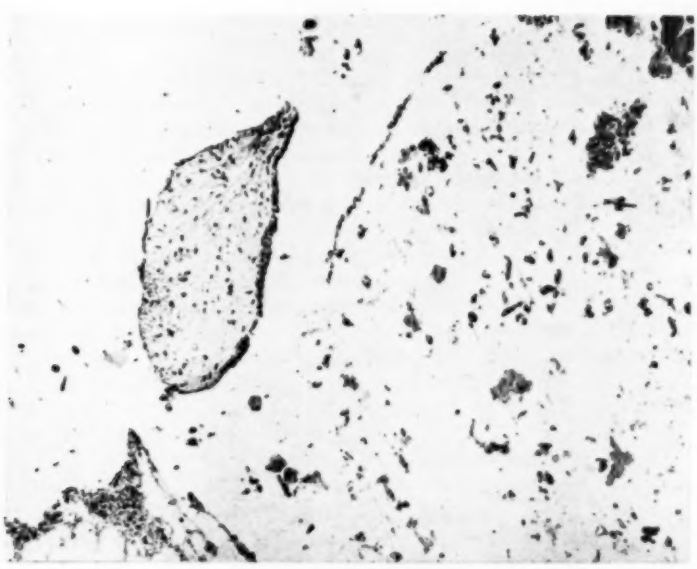
The term is used to describe undifferentiated carcinomas which are not oat-cell tumours, where the amount of tumour in the biopsy tissue is considered adequate to make a reasonable assessment of cell type. Similar tumours have been described by others as polygonal cell, spheroidal cell, large cell, and undifferentiated carcinoma. Many "clear cell" carcinomas are included in this group.

EXPLANATION OF PLATE

FIG. 1.—Section of centrifuged deposit from second wax bath, which had been used for two runs of the processer. The larger fragments present show an areao f omental fat necrosis, a fibrosed chorionic villus, and a necrotic fragment of carcinoma. H. and E. ×62.

FIG. 2.—High power view of necrotic carcinoma in right upper corner of Fig. 1. H. and E. ×340.

FIG. 3.—Open wire-mesh cage, showing biopsy tissue wrapped in linen and operation specimen carcinoma block wrapped in gauze.



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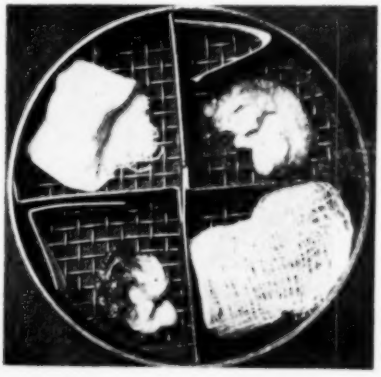

3

I prefer the word "simplex", which does not specify the shape of the cells and means "simply carcinoma".

Carcinoma

The unqualified word "carcinoma" is used when the amount of tumour present is too small to expect to be able to see evidence of differentiation, or to say more than that carcinoma is present.

In some cases this is because of inadequate biopsy and sometimes because of extensive tumour necrosis. In about a quarter of biopsies where this diagnosis is made it is because the tumour lies distal to the biopsy site and all that can be seen in the specimen are carcinoma cells or cell clumps in the sub-epithelial lymphatics of otherwise normal bronchi.

Though different as regards the names of cell types, this classification is similar to the ones used in some other centres; by Walter and Pryce (1955), Raeburn and Walter (1956), and Hinson (1958) in London, by Nicholson *et al.* (1957) in Manchester, by McDonald *et al.* (1951) in the United States, and by Kreyburg (1954, 1959) and Höst (1960) in Norway. Statistics relating to lung carcinoma cell types from these centres should be comparable.

SURVEY

Benign Tumours (Table II)

TABLE II.—*Analysis of Benign Tumours*

Adenomas	26
Papillomas	5
Fibromas	3
Lipomas	2
Chondroma	1
Amyloid tumour	1
Polyps	2

Bronchial adenomas

Except for one patient who was unfit for surgery, all had the biopsy diagnosis confirmed in later operation specimens. There were 24 carcinoid adenomas and 2 cylindromatous adenomas. From the biopsy specimens it was possible to classify 2 tumours as "malignant carcinoid adenomas" and 2 as infiltrating carcinoid adenomas, features confirmed in later operation specimens. Eight other cases proved to have infiltrating adenomas, and one other had a hilar lymph node involved with a secondary "bronchial adenoma" in the liver when the biopsy specimens had shown the most innocent-looking carcinoid adenomas.

Bronchial adenomas represent only about 2 per cent of the neoplasm diagnosed in this series. The sex ratio shows a 1·4 : 1 male predominance, and the age distribution is much wider than with the carcinomas (Table III).

Table III.—*Adenomas*

Sex and age at biopsy		
Under 40 years	40–60 years	Over 60 years
8 cases	10 cases	8 cases

Male	Female
15	11

Bronchial papillomas

These rare tumours are described as usually occurring as a multiple condition of the upper respiratory tract in children (Liebow, 1952).

Five cases of isolated bronchial papilloma occurred in this series, one in a woman of 50 years of age, and the others in men aged 52 years, 52 years, 53 years and 62 years. All the tumours were small and pedunculated and arose from lobar or segmental bronchi. In two cases the patients also had squamous carcinomas in central bronchi, but never in the same lobe as the papilloma.

Microscopically these lesions show polypoid tumours composed of non-ciliated respiratory epithelial cells with some mucous cysts among the cells. The tumours have almost no fibrous core and they are covered by intact epithelium.

In another case, a man aged 30 years with rapidly increasing dyspnoea and coarse mottling throughout both lung fields, the bronchoscopic description was " diffuse fine papillomatous lesions of the right main and upper lobe bronchus ". The section showed delicate papilliferous lesions covered by columnar epithelium, but at post mortem soon afterwards it was found that the patient had advanced malignant adenomatosis, with tumour deposits filling the sub-epithelial lymphatics of the larger bronchi and extruding as papillomas through the intact respiratory epithelium.

Bronchial fibromas

The degree of malignancy of these tumours remains uncertain, and they might better be classified as fibrosarcomas.

They all arose in lobar bronchi from broad bases, though the tumours were never over 8 mm. across. Microscopically they showed a cellular but well-differentiated fasciculated fibromatous pattern and appeared to arise from sub-epithelial connective tissues of the bronchi. They were partly covered by respiratory epithelium.

In the first case, a girl aged five years, in spite of good differentiation the tumour cells showed some irregular hyperchromatic nuclei and more mitoses than is usual in benign connective tissue tumours, so it was called a low grade fibrosarcoma. The operation specimen showed that the bronchial tumour was quite superficial, but tumour deposits were present in one hilar lymph node.

The other two cases were a boy aged ten years and a man of 51 years of age. The histology of these cases was similar to the first one, but there was no lymph node involvement.

Other benign tumours

These all arose in large bronchi, but showed no unusual histological features.

The bronchial polyps are not really tumours, and in basic structure are similar to laryngeal or cervical polyps, with abundant stroma covered by hyperplastic epithelium.

The case of bronchial amyloid tumour has already been reported (Whitwell, 1953).

Malignant Tumours

From the case records of the patients it has been possible to divide the malignant tumours in the series into primary lung carcinomas, recurrences, second biopsy specimens and secondary tumours.

The secondary tumours were mainly from carcinomas of the oesophagus, breast and kidney, with a few soft tissue sarcomas. The figures given in Table IV are derived from the reviewed section reports for 1950 to 1955 inclusive, and from the original reports for 1956 to 1959 inclusive.

TABLE IV.—*Analysis of Malignant Tumours*

Total	1368
Primary lung carcinomas	1329
Second biopsies	4
Recurrences	15
Secondary tumours	20

Analysis of 1329 *Primary Lung Carcinomas*

Squamous carcinoma (including 10 reports of Bowen's disease)	554	Approximately 42 per cent
Oat-cell carcinoma	447	33·5 per cent
Carcinoma simplex	219	16·8 per cent
Adenocarcinoma	27	2 per cent
Adenomatosis	3	—
Mixed cell types	19	1·4 per cent
Carcinoma	60	6 per cent

Incidence of bronchial carcinoma

In the first half of the survey period the Regional Thoracic Surgical Centre at Broadgreen Hospital was the main centre for diagnostic bronchoscopy in the Liverpool, North Wales and Isle of Man area, the same area for which the Liverpool Cancer Control Organisation keep records of all notified malignant disease. In this period, the lung carcinomas occurring in the survey represent 21 per cent of those notified to the L.C.C.O.

In the second half of the survey period two other large diagnostic bronchoscopy centres were working in Liverpool, and the carcinomas in the survey during this time represent only 11 per cent of cases notified to the L.C.C.O., indicating that only half the patients then went to Broadgreen.

In spite of having only half the patients in this second period only 6 per cent fewer carcinomas were diagnosed.

TABLE V.—*Comparison of Original and Review Histology Reports* (1950–55)

Number of carcinoma reports	Number altered in review
807	64 (8 per cent)

Year	Percentage of reports altered in review
1950	19
1951	7·3
1952	6
1953	7
1954	6
1955	3

Alterations made in review, by cell type

	Squamous	Oat-cell	Simplex	Adenocarcinoma
Number of alterations	18	5	41	0
Percentage of reports altered	5·7	1·9	31	0

Review of 1950 *to* 1955 *Histology Sections* (Table V)

It doesn't follow that the review cell-typing is more correct than the original assessment, but the table shows the value of standardisation of nomenclature.

Nineteen per cent of carcinomas from 1950 showed a different type when reviewed, but after standardisation in 1951 the numbers of differing reports fell to 6–7 per cent, and when only two pathologists were concerned this figure fell to only 3 per cent.

Table V also shows the variations in reporting that occurred with each cell type. There is rarely any doubt about the nature of an adenocarcinoma or oat-cell tumour, there is a small inconsistency in reporting squamous carcinoma, but there is considerable variability in the interpretation of carcinoma simplex. In the whole period nearly a third of the biopsy carcinoma simplex diagnoses had to be altered, but a lot of this was accounted for in 1950 when oat-cell tumours were sometimes called carcinoma simplex.

Doubtful Reports and False Negative Reports

The overall figure of 62 per cent positive biopsy for the series means very little and cannot be compared profitably with any other series ; because many patients did not have carcinomas, some had peripheral tumours, and biopsy specimens were not taken at bronchoscopy from all visible tumours when the condition was considered to be untreatable.

Though it was not possible to find out accurately how many of the 1445 patients of the 1950–55 period really had lung carcinoma, a search of the L.C.C.O. records up to the end of 1958 showed that 937 of the patients were eventually registered as lung carcinoma, usually with histological confirmation or on sound clinical evidence with the inevitable fatal outcome, which would imply 86 per cent positive diagnosis. As some cases must have escaped registration, it is probable that about 20 per cent of the patients in the series with negative bronchial biopsy actually had lung carcinoma.

Among the 1445 biopsy sections of the 1950–55 period the original reports showed 783 (54 per cent) carcinoma, 24 (1·7 per cent) probable carcinomas, and 14 (1 per cent) possible carcinomas. When these sections had been re-examined there were 807 (56 per cent) carcinomas, 23 (1·6 per cent) probable carcinomas and 19 (1·3 per cent) possible carcinomas. Sections diagnosed as " probable " in the original reports nearly all became definite carcinomas in the review, and sections originally " possible " nearly all became probable on review. In addition, ten new carcinomas were seen in the review where the previous reports had been normal.

One cannot attach to much importance to the extra 2 per cent carcinomas found in the review. The reviewer has a dispassionate objectiveness in making a diagnosis which makes him bolder to diagnose malignancy than the pathologist reporting originally, who has a much greater responsibility. However it was clear that at times undue caution was originally shown in differentiating streaming oat-cells from squashed lymphocytes nipped by biopsy forceps, and in diagnosing lung cancer when only tumour cells in sub-epithelial lymphatics were present in the sections.

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Comparison of Operation Specimen Histology with Bronchial Biopsy Histology
(Table VI)

The biopsy reports used for this comparison were the review reports up to the end of 1955, thereafter the original reports.

TABLE VI.—*Comparison of Biopsy and Operation Specimen Histology Reports*

Type	Number of cases	Biopsy different from operation histology report	
		Number	Approximate percentage
Adenoma	25	0	0
Squamous carcinoma	177	3	1·7
Oat-cell carcinoma	69	1	1·5
Carcinoma simplex	65	20	31
Adenocarcinoma	8	0	0
Total	344	24	7

Assuming that the main specimen report is correct, because so much more tissue is examined histologically, it can be seen that there is only a small error in diagnosing adenocarcinoma, oat-cell carcinoma and squamous carcinoma in biopsy specimens, but there is considerable error in diagnosing carcinoma simplex, which may eventually turn out to be a squamous carcinoma or an adenocarcinoma.

Only eight adenocarcinomas were available for comparison, because being nearly always a peripheral tumour they are rarely found in bronchial biopsy specimens, and when they reach the large bronchi they are usually inoperable.

Collation of Case Records with Histology

No false positive report of carcinoma was known to have occurred in the series and it is improbable that any surgical colleague would fail to draw the attention of the pathologist to any positive biopsy report where later surgery produced a non-malignant specimen, or where an untreated patient showed unconscionable survival!

As a further check, all L.C.C.O. records of positive biopsy patients diagnosed up to the end of 1958 have been examined. The files show that carcinoma was present in all patients submitted to major surgery and that all patients diagnosed by the end of 1955 but not given treatment were dead by 1958. At the time of this check it was still too early to know the fate of all patients diagnosed after 1955.

It was found also that all patients diagnosed as "probable" carcinoma, both in the original and review reports, before the end of 1955 were dead by 1958, except for those in whom the diagnosis had been confirmed by operation specimen histology. Some "possible" carcinomas were also registered as lung cancer on clinical grounds, but most of this group could not be traced.

However, there are occasional odd cases where the histology has had to be re-examined with some anxiety. The following case is an example—a man aged 50 years was bronchoscoped in May 1954 as he had been spitting blood for a few weeks, and a plague of tumour was seen in the right lower lobe bronchus just above the apical branch orifice. Section showed this to be a squamous carcinoma and the case was considered operable, but the patient refused all treatment. He recovered his health and was re-examined in May 1955, 1956 and 1957 and found to be in

good health. He died at home in October 1957 in a 'flu epidemic and was brought to hospital for post mortem examination, which showed that death was due to influenzal pneumonia with staphylococcal lung abscesses. No carcinoma was seen on first looking at the lungs, but after consulting the bronchoscopy notes a small thickened area was found in the lower lobe bronchus, which proved to be a squamous carcinoma. No metastases were found.

DISCUSSION

Though the survey has not shown accurately how many central lung carcinomas were missed by the bronchoscopist or pathologist, it has shown that about 20 per cent of lung cancer cases who had bronchial biopsy performed had negative findings. No false positive reports were made and the uncertain reports amounted to 3·2 per cent of biopsies.

It may be that the absence of false positives reflects excessive caution in reporting malignancy, a policy which might also produce a higher proportion of false negative reports. However, consideration of the natural history of lung cancer, treated and untreated, and of the risks of surgery, makes a cautious approach advisable.

It has also been shown that a considerable measure of consistency in reporting cell types of carcinoma can be achieved by preserving rigid definitions of cell types and by creating a heterogeneous type to include all doubtful cases. By doing this, the actual numbers and percentages of the defined types in any series is reduced but this is more than compensated for by the higher degree of accuracy achieved, which may be of value in studying group biological characteristics of the defined types. Both the review of the biopsy series and the comparison with operation specimens show that the cell-type inconsistencies in reporting squamous carcinoma is under 6 per cent, in oat-cell carcinomas it is under 2 per cent, and no errors were found with the small number of adenocarcinomas seen.

Many writers claim that a higher proportion of positive results can be obtained by studying sputum cytology than by bronchial biopsy, and it is sometimes said that all suspected lung carcinoma patients should have a cytological examination of their sputum (Farber *et al.*, 1950 ; Osborn, 1953). Very few patients in the Thoracic Centre at Broadgreen Hospital have cytological examinations of sputum, usually only those with doubtful peripheral shadows and negative bronchoscopic findings.

As sputum cytology examinations must not be made for at least a week after bronchoscopy, to avoid getting false positive results (Philps, 1954), the best time to do these tests is before bronchoscopy To say that the tests should be done before admission to hospital would be to cancel all the efforts that have been made to get suspected patients quickly to diagnostic centres.

Surgeons rightly do not wish to delay for several days after admission making what is usually at bronchoscopy a precise clinical and histological diagnosis and assessment of operability. Such delay crowds the words, postpones surgery and allows time for colonisation of patients with antibiotic-resistant staphylococci which will be an added hazard if surgical treatment is to be attempted. Pathologists in surgical thoracic centres appreciate this attitude and usually have no time for the numbers of sputum cytology examinations which would be required if this test became part of the routine investigations of lung cancer.

In the ten year period surveyed about 8000 patients were investigated for suspected lung cancer. Cytodiagnosis requires for each case a minimum of three slide examinations, each taking about ten minutes, so cytodiagnosis would have taken about 4000 hours. This would have occupied a whole-time pathologist working on nothing else almost exactly two years, in the course of which he would have produced about 150 false positive reports and many more doubtful ones.

In my opinion cytodiagnosis of sputum should be reserved for patients suspected of lung cancer who have negative bronchoscopic findings, or are considered unsuitable subjects for surgery. The main value of cytodiagnosis of central tumours is to provide necessary practice for the pathologist and this can be obtained better by examining sputum from inoperable lung cancer patients in medical wards.

SUMMARY

The histology of 2310 bronchial biopsies examined in a ten year period has been surveyed with a brief description of the benign tumours found and a more detailed report of the carcinomas.

The accuracy of the histological diagnosis of lung cancer has been checked by re-examining the slides of the first six years of the survey period and comparing the findings with the original reports, by comparison of the biopsy histology with that of operation specimens from the same cases and by a follow-up of all cases. Carcinoma was reported in 62 per cent of all biopsy specimens and about 65 per cent of the patients in the biopsy series eventually were found to have lung cancer. No false positive reports occurred.

The accuracy of carcinoma cell-typing was checked by the same re-examination of the slides of the first six years and by comparison of biopsy with operation specimen reports. It was shown that the discrepancy in reporting squamous carcinoma was under 6 per cent, oat-cell carcinoma under 2 per cent, and no variation was found with adenocarcinoma. Carcinoma simplex was the most variable type reported, 31 per cent being given a different histological type when the operation specimens were examined.

REFERENCES

Evans, R. W.—(1956) ' Histological Appearances of Tumours '. Edinburgh (Livingstone), p. 722.

Farber, S. M., Rosenthal, M., Alston, E. F., Benioff, M. A. and McGrath, A. K.—(1950) ' Cytological Diagnosis of Lung Cancer '. Springfield (Thomas), p. 5.

Hinson, K. F. W.—(1958) in ' Carcinoma of the Lung '. Ed. Bignall, J. R. Edinburgh (Livingstone), pp. 115–123.

Höst, H.—(1960) *Cancer*, **13**, 1167.

Hutchison, H. E.—(1952) *Ibid.*, **5**, 884.

Kreyberg, L.—(1954) *Brit. J. Cancer*, **8**, 199.—(1959) *Acta Un. int. Cancr.*, **15**, 78.

Liebow, A. A.—(1952) ' Tumours of the Lower Respiratory Tract: Atlas of Tumour Pathology '. Washington (Armed Forces Institute of Pathology), p. 22.

McDonald, J. R., McBurney, R. P., Carlisle, J. C. and Patton, M. M.—(1951) *J. thorac. Surg.*, **22**, 62.

Nicholson, F., Fox, M. and Graham Bryce, A.—(1957) *Lancet*, i, 296.

Osborne, G. R.—(1953) ' Applied Cytology '. London (Butterworth & Co. Ltd.), p. 3.

Phelps, F. R.—(1954) *Brit. J. Cancer*, **8**, 67.

Raeburn, C. and Walter, J. B.—(1956) *Lancet*, i, 778.

Walter, J. B. and Pryce, D. M.—(1955) *Thorax*, **10**, 107.

Whitwell, F.—(1953) *Ibid.*, **8**, 309.

THE HISTOPATHOLOGY OF LUNG CANCER IN LIVERPOOL: THE SPECIFICITY OF THE HISTOLOGICAL CELL TYPES OF LUNG CANCER

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PATHOLOGISTS occasionally see lung cancer where the primary tumour is of one cell-type, and a secondary tumour deposit is of a different pattern. They see also primary tumours where the cell-types are mixed, and primary tumours of one cell-type with secondary deposits in hilar lymph nodes of a different type. However, these are the exceptions, for usually the cell-type remains constant throughout the primary tumour and all its secondary deposits.

The rate of occurrence of mixed cell types in lung cancer has been a debated topic for many years and it is one of the main themes of this paper.

Willis (1960), in studying 84 post mortem cases, found that only one cell-type was present in 77 per cent of lung cancers, the rest of the tumours being mixtures of two or more patterns. He said " to set out, for example, to label all bronchial carcinomas either 'squamous-celled', 'oat-celled' or 'adenocarcinoma' is both futile and impossible. The entity is bronchial carcinoma and all other prefixes or adjectives are merely descriptive of the possible variations of structure seen in tumours, or a tumour, of that species." This thesis, that the entity is lung cancer and not the cell-type, has claimed support from the wide variation in the frequencies of cell-types in many large series of cases reported in the literature, for it has often been argued that there should be some constancy in the frequencies of cell-types in large series if the typing indicated any more than the pathologist's bias or the random selection of blocks of tumour for study (Anderson, 1948). Barnard (1926), who originally described the oat-cell carcinoma, said that if enough tumour is examined several cell-types may often be found and similar conclusions were reached by Phillips, Basinger and Adams (1950) using large histological sections of lungs. Budinger (1958) reported that 80 per cent of 250 post mortem cases of lung cancer showed mixed cell-types.

However, Walter and Pryce (1955) have accounted for some of the variations in reported cell-type frequencies. They examined very thoroughly 207 operation specimens and 139 post mortem specimens of lung cancer, using the same histological criteria for grading the types in both series, and they found quite different cell-type frequencies in these series—for example, squamous tumours formed about 60 per cent of the operation specimens but only 20 per cent of the post mortem cases. They considered that the post mortem series represented the true incidence of the cell-types in lung cancer. Walter and Pryce (1955) showed that oat-cell carcinoma can have an adenocarcinomatous pattern in some parts of the tumour (confirmed by Azzopardi in 1959), and that squamous metaplasia can occur in any lung cancer, facts which if not appreciated lead to a diagnosis of mixed-

cell carcinoma. They found no true mixed-cell tumours among their cases and concluded that lung cancer is not so pleomorphic that classification is valueless.

In the last decade many analyses of large series of cases of lung cancer have been published which, though mainly concerned with the results of surgical treatment, have also tabulated the age, sex, symptoms, and survival of patients with different histological types of carcinoma (Mason, 1949 ; Bignall and Moon, 1955 ; Gifford and Waddington, 1957 ; Nicholson *et al.*, 1957 ; Bignall, 1958). Unfortunately, in many accounts the histological reports have been made by several pathologists and have not been reviewed ; it is not always clear whether the histology is based upon bronchial biopsy, secondary tumour biopsy, operation specimen, or post mortem specimen ; and often the oat-cell tumours have been included with the undifferentiated or anaplastic carcinomas. Usually pathologists (Walter and Pryce, 1955 ; Hinson, 1958 ; Mullaney, 1958 ; Azzopardi, 1959) consider oat-cell carcinoma to be an entity and describe its histological features, but when making statistical analyses the clinicians (Mason, 1949 ; Bignall and Moon, 1955 ; Gifford and Waddington, 1957) have included oat-cell tumours with other undifferentiated carcinomas, so that the biological features of oat-cell carcinoma are masked by the other undifferentiated tumours in the group, and are not appreciated. Only a few surveys (McBurney, McDonald and Clagett, 1951 ; Kreyberg, 1954, 1959 ; Nicholson *et al.*, 1957) treat oat-cell carcinoma as an entity ; but even without much statistical evidence many surgeons believe the oat-cell carcinoma to be a distinctive and particularly malignant form of lung cancer. At a recent symposium on Cancer of the Lung Mason (1960) quoted Dr. O. T. Clagett as saying " the small-cell or oat-cell carcinoma has a prognosis so poor that perhaps it should not be operated upon at all ".

During the investigation of case records and Liverpool Cancer Control Organisation records, which was part of the review of 1329 lung carcinomas diagnosed by bronchial biopsy during 1950 to 1959, reported in the preceding paper (Whitwell, 1961), opportunity was taken to study some biological features of the different cell-types of lung cancer, the definitions of which are given in the preceding paper. Special attention was given to the squamous and oat-celled carcinomas, as these cases were numerous and considered to be accurately typed.

After an optimistic start on a larger scale, the number of factors studied was reduced to three, where there was nearly always accurate objective evidence. These three were the sex, age and survival of the patients. Accurate information about duration and nature of symptoms and smoking habits, was not always available and most of the patients were dead when the survey was made. As most of the patients had inoperable tumours and few came to post mortem examination it was not possible to know the exact site or size of the tumours.

To obtain a wider approach to the significance of cell-types in lung cancer, for comparison with the biopsy cases, I have also studied an operation specimen series and a post mortem specimen series, where the tumours had been examined and typed by the same pathologists using the same histological criteria as in the biopsy series, and where the patients came from the same geographical region in the same period of years. The operation and post mortem series have the advantage that more tumour tissue was studied in each case, rarely less than four blocks being examined histologically, so the diagnosis of carcinoma simplex was more accurate in these series, which also provided far more adenocarcinomas than the biopsy series. In the operation specimen series there was usually accurate information

about the size and site of the tumours and the extent of lymph node involvement. The post mortem material provided cases where full clinical records were available and where the extent of distant metatases had been noted.

Another series briefly mentioned, consists of the lung cancers which were revealed during the Liverpool Mass Radiography Campaign of 1959.

TABLE I.—*Bronchial Biopsy Series*

Aneysis of 1329 lung cancer cases		
		(per cent)
Squamous carcinoma	554	42
Oat-cell carcinoma	447	33·5
Carcinoma simplex	219	16·8
Adenocarcinoma	27	2
Adenomatosis	3	0·23
Mixed cell types	19	1·4
Other carcinomas	60	4·5

Biopsy series (Table I)

This consisted of the 1329 primary lung cancers reported between 1950 and 1959, described in the preceding paper.

TABLE II.—*Operation Specimen Series*

Analysis of 907 specimens		
		(per cent)
Squamous carcinoma	485	54
Oat-cell carcinoma	140	15·2
Carcinoma simplex	152	16·9
Adenocarcinoma	90	10
Adenomatosis	9	1
Mixed cell types	26	3
Other carcinomas	5	5·5

Operation specimen series (Table II)

These were all the main operation specimens of lung cancer examined for the Regional Thoracic Surgical Centre at Broadgreen Hospital, Liverpool, in the same ten year period as the biopsy series. There is some overlap of cases in these two series, because about a third of the operation cases had had a positive bronchial history in Broadgreen Hospital and were therefore included in both series. Another third of the operation cases had had a positive biopsy in some other hospital and about a third had had no biopsy or a negative biopsy.

TABLE III.—*Post Mortem Specimen Series*

Analysis of 128 cases		
		(per cent)
Squamous carcinoma	18	14
Oat-cell carcinoma	52	41
Carcinoma simplex	14	11
Adenocarcinoma	35	27·5
Adenomatosis	2	1·6
Mixed cell types	7	5·5

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TABLE IV.—*Post Mortems on General Medical and Surgical Ward Patients 1950–1959*

	Total PMs.	Total cancer	Lung cancer		Other cancer	
			Number	% of PMs.	Number	% of PMs.
1950–1954	1053	194	50	4·7	144	13·8
1955–1959	1389	283	78	5·7	205	14·8
1950–1959	2442	477	128	5·3	349	14·2

Post mortem specimen series (Tables III and IV)

The aim has been to produce a series which is comparable with that of any other acute general hospital and representative of the adult population of Merseyside—the same population which has largely produced the biopsy and operation specimen material. For this reason the survey included all post mortem examinations held in Broadgreen Hospital in the same ten year period, but excluding all deaths occurring in the Thoracic Surgical Centre and in the Maternity Department, and all accident deaths investigated for the Coroner. The hospital has a roughly equal number of beds for men and women and there are no wards for children.

Significance of Sex and Cell-type

Lung cancer in all three series showed a marked male predominance, with an overall M : F ratio in the biopsy series of 8·4 : 1, the operation series ratio being 10·4 : 1, and the post mortem series ratio being 3·9 : 1.

In this post mortem series as in most others (Christiansen, 1953 ; Jakobsen, 1953 ; Galluzi and Payne, 1955) the proportion of women is higher than in clinical series, probably mainly for the social reason that, when they are ill, fewer women than men are able to find someone to look after them in their homes. However, there is a close linkage between the different M : F ratios found and the cell-types which composed the three series.

Squamous carcinoma

This is most predominantly a tumour of men, in whom it accounts for 44·5 per cent of central tumours. In women only 17 per cent of the central tumours were of squamous type—in fact experience has shown that when a biopsy reveals squamous carcinoma in the bronchus of a woman there is considerable chance that it is from a primary oesophageal growth.

The three series showed the following M : F ratios for squamous carcinoma—biopsies 22 : 1, operation specimens 23 : 1, and post mortem specimens 17 : 1. The ratios come between the 11·4 : 1 quoted by Bignall (1958) and the 26·4 : 1 found by Doll and Hill (1952).

Oat-cell carcinoma

In biopsy specimens this was the commonest tumour found in women, and the second commonest found in men, the M : F ratio being 4·1 : 1. The ratio in the operation series was 3·3 : 1, and in the post mortem series it was 3 : 1.

The relative commonness of this carcinoma in women has been largely overlooked, through the inclusion in most series of oat-cell tumours with other un-

differentiated growths. However, Nicholson and his colleagues (1957), in Manchester, thought that the poor prognosis and resection rate in women might be due to the high proportion of them with oat-cell tumours, and Hinson (1958) reported from London the M : F ratio of resected oat-cell carcinomas as 2·3 : 1.

Adenocarcinoma

The operation specimen series contained the largest number of these growths, the M : F ratio being 5·9 : 1. In the 27 cases in the biopsy series the ratio was 5·4 : 1 and in the post mortem cases it was 3·3 : 1.

These findings agree with many reports which mention the relative commonness of adenocarcinoma in women (Liebow, 1952 ; Bignall, 1958 ; Budinger, 1958).

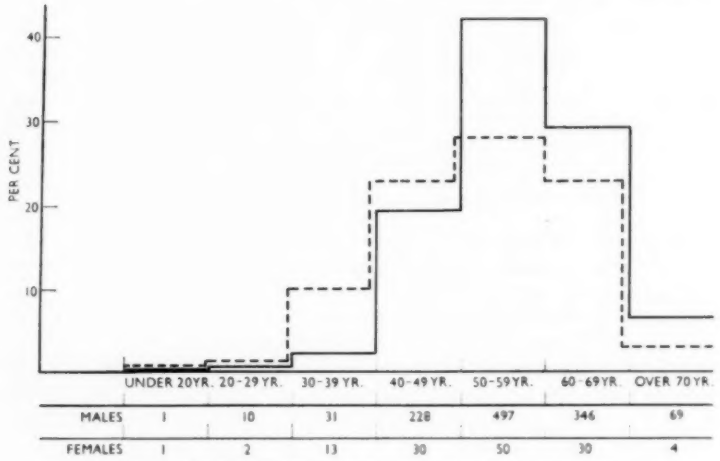

FIG. 1.—Age distribution of lung cancer cases in biopsy series.
Male ———— Female - - - - - -

Carcinoma simplex

The 152 specimens in the operation series were the most accurately typed of this group and the M : F ratio was 14 : 1. The ratio in the biopsy series was 17 : 1 and in the post mortem series it was 5·5 : 1.

Age Distribution and Cell-type

The overall age distribution of lung cancer in men and in women is shown in Fig. 1, which was derived from 1312 patients in the biopsy series, the age of the 17 other patients in this series not being known. In both sexes lung cancer occurred most commonly in the fifth decade, the highest incidence being between 55 years and 60 years.

The age distribution is much more widely scattered in women, in whom cancer occurred at a rather earlier age than in men. In men only 23 per cent of tumours appeared before the age of 50 years, whereas in women 35 per cent of carcinomas

had occurred by that age. Also, in men 35 per cent of tumours were found in those over 60 years of age, whereas in women only 26 per cent of cases occurred in this age group.

The earlier appearance of lung cancer in women than in men can be largely explained by studying the cell-type distribution in the two sexes and by examining the age distribution tables of different cell-types of lung cancer, both in men and women. Fig. 2 and 3 show the age distribution of squamous carcinoma and oat-cell carcinoma in both men and women, obtained from the biopsy series.

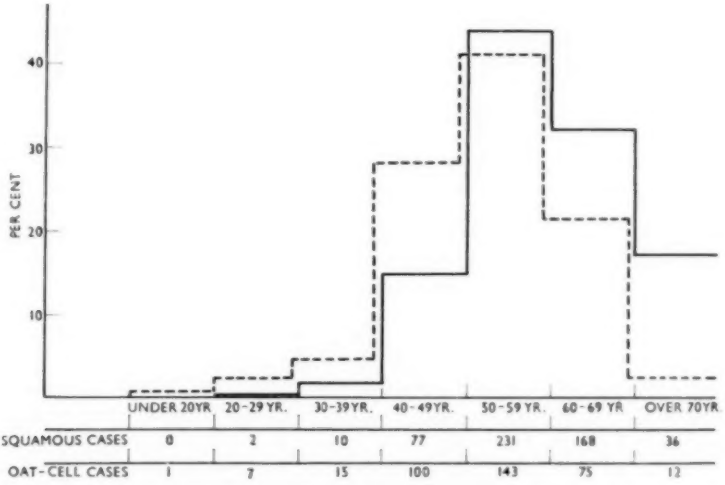

FIG. 2.—Age distribution of male lung cancer cases in biopsy series.
Squamous carcinoma ———— Oat-cell carcinoma - - - - - -

In men 35 per cent of oat-cell tumours but only 17 per cent of squamous tumours appeared before the age of 50 years. In women 43 per cent of oat-cell tumours but only 21 per cent of squamous tumours were found in those under 50 years of age. The similar male and female age distribution tables of these two types of lung cancer suggests that it is the different cell-type distribution in the two sexes which is the main cause of the earlier appearance of lung cancer in women. A somewhat similar finding was reported by Umiker and French (1960), who found that 25 per cent of the oat-cell carcinoma patients were under 50 years of age, while only 2·6 per cent of squamous tumours were from this age group. All their cases were male. Kreyberg (1959) found no difference in the age distribution of squamous carcinoma and oat-cell carcinoma.

The age distribution tables for carcinoma simplex and adenocarcinoma are shown in Fig. 4, the figures being derived from the operation specimen series and they are similar to squamous carcinoma, with between 17 and 20 per cent of tumours producing symptoms before the age of 50 years.

The only type of lung cancer in these 1312 patients to show an unusual age distribution was the oat-cell carcinoma.

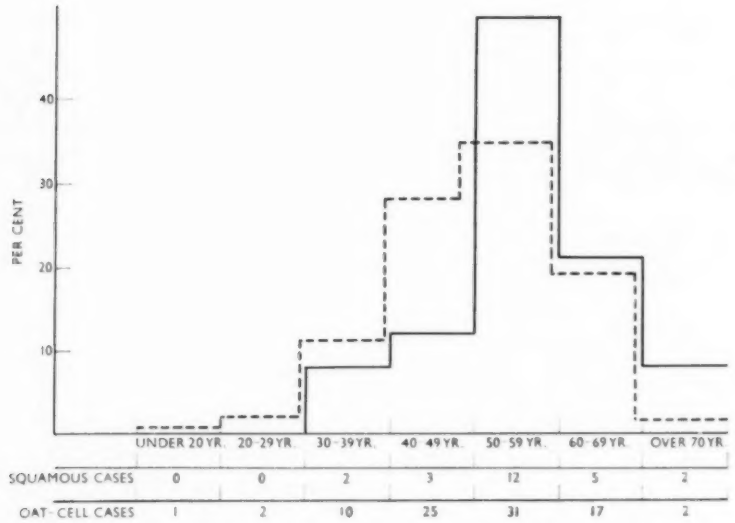

FIG. 3.—Age distribution of female lung cancer cases in biopsy series.
Squamous carcinoma ———— Oat-cell carcinoma - - - - - -

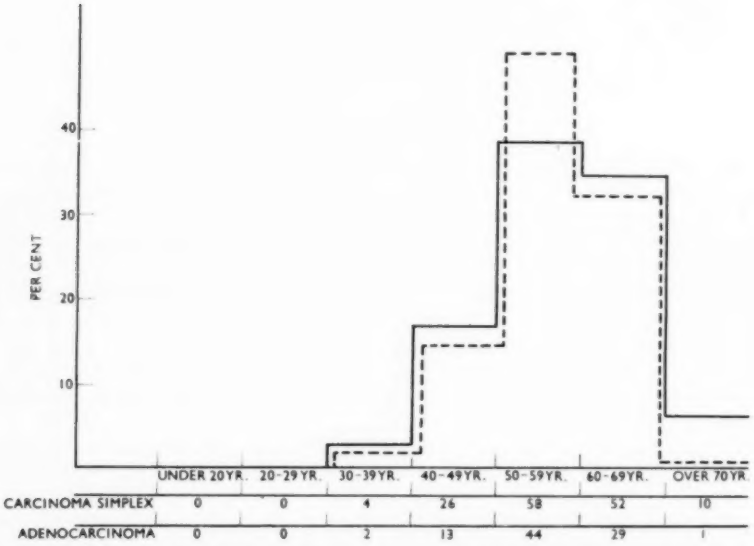

FIG. 4.—Age distribution of lung cancer cases in operation specimen series.
Carcinoma simplex ———— Adenocarcinoma - - - - - -

Survival and Cell-type

There are many accounts of the survival rates after radical surgery and after radiotherapy of lung cancer patients of different cell-types. Many of the patients in the present series were included in the account by Gifford and Waddington in 1957, who found that the survival rate of surgically treated patients with squamous carcinomas was better than that of patients with undifferentiated carcinomas (including the oat-cell tumours), which agreed with the findings of Bignall and Moon (1955).

However, such surveys do not necessarily represent the biological behaviour of the cell-types, because of the interference by the surgeons and radiotherapists, and it was thought worthwhile to find out the survival rates of untreated patients.

In the present biopsy series the largest accurately typed groups were the squamous and oat-cell carcinomas and the survival of patients with these tumours has been studied where the patients received no treatment other than blood transfusion or sedation. The usual reason for lack of other treatment was the advanced stage of the disease, but sometimes it was because of the poor general condition of the patients and occasionally because treatment was refused. The patients studied were those diagnosed in the 1950–1955 period and their records were followed, when necessary, up to the end of 1958. There were 419 patients including 147 with squamous carcinoma and 202 with oat-cell carcinoma. The survival rates of these patients (Fig. 5) show that those with squamous tumours lived considerably longer than those with oat-cell tumours. Three months after diagnosis 41 per cent of patients remained alive, including 56 per cent of those with squamous tumours but only 38 per cent of those with oat-cell tumours. After six months these percentages had fallen respectively to 35 per cent and 12 per cent, and after a year 18 patients remained alive, including 15 with squamous tumours but only one with oat-cell carcinoma.

These results agree with Lea (1952) who found that the mean duration of life in untreated lung cancer was 10·8 months with squamous carcinoma, and only 5·2 months with " anaplastic " carcinoma.

Tumour Size, Lymph Node Involvement and Cell-type

This information (Table V) was obtained from specimens in the operation series, where the average widest cross section of tumours in the fixed specimens had been measured and lymph nodes attached to specimens and sent separately by the surgeons had been examined histologically for tumour deposits.

The tumours have been divided into small and medium sized ones (under 40 mm. diameter) and large tumours (over 40 mm. diameter). In the small and medium sized group were two thirds of the squamous carcinomas, half the carci-

TABLE V.—*Size of Tumours and Lymph Node Involvement in Operation Series*

	Number	Tumours under 40 mm. diameter (per cent)	Lymph node involvement (percentage)	
			under 40 mm.	over 40 mm.
Squamous carcinoma	485	67	33	35
Carcinoma simplex	152	60	40	42
Oat-cell carcinoma	140	55	50	66
Adenocarcinoma	90	58	27	50

noma simplex specimens and just over half the adenocarcinomas and oat-cell carcinomas.

In about a third of the squamous tumours some lymph nodes were involved by growth, and this proportion did not depend much upon the size of the primary

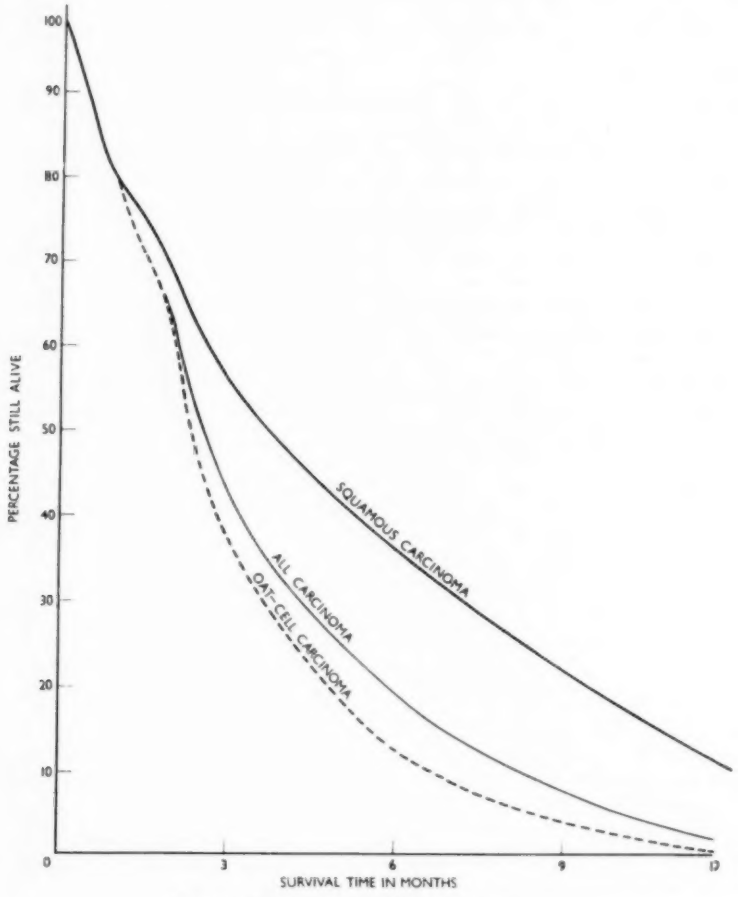

FIG. 5.—Survival with untreated lung cancer.

tumour. Carcinoma simplex had rather more lymph node involvement than squamous carcinoma, but similarly, this did not appear to depend much on the size of the primary growth. Oat-cell tumours had the highest lymph node involvement of the two groups of tumours. Small adenocarcinomas had less lymph node involvement than any other carcinomas but large adenocarcinomas had more involvement than any tumours except oat-cell carcinoma.

This series shows a much lower percentage of lymph node involvement than has been reported by Hinson (1958), probably explainable by his more thorough examination of lymph nodes. However, Hinson also found least involvement (65 per cent) in squamous carcinomas and most (90 per cent) in oat-cell tumours.

POST MORTEM SERIES

Before describing histological aspects of this series it is worthwhile considering whether or not it is justifiable to regard post mortem cell-type statistics as typical of lung cancer as a whole.

Steiner (1953) states "autopsy material is the type of study at the present time that can reveal all of the lung cancer in a population" and he implies that the cell-type frequencies found in a post mortem series are a true mirror of lung cancer. Sellors (1955) and Walter and Pryce (1955) express the same opinion.

However, Willis (1960), discussing the influences which can affect the incidence of neoplasms in a post mortem series, considers that the percentage of post mortems held on hospital deaths and the availability of beds for the chronic sick to take inoperable cases of malignant disease, affect the percentage of different neoplasms in any series. Also, when post mortems are performed infrequently they tend to include a high proportion of cases of undiagnosed neoplasms and of cases where symptoms were due to metastatic deposits.

The present post mortem series demonstrates the truth of Willis' statement and shows how not only the incidence of lung cancer but also the cell-type frequencies found in lung cancer in a post mortem series are dependent upon influences which are not entirely of a medical nature.

In the period of the survey post mortems were held on 43 per cent of hospital deaths, and the hospital record of death certificates shows that post mortems were held on 41 per cent of patients registered as dying from lung cancer.

Post mortem examination permission is requested by administrative staff, usually on the advice of the medical staff and also directly by the medical staff. When post mortems are held the death certificates are usually completed after the post mortem result is known. Facilities do exist at the hospital for transferring at least some cases of inoperable carcinomas to hospitals for the chronic sick.

These factors mean that there is some ante mortem selection of cases, by transfer of diagnosed inoperable patients; and also considerable selection later, due to the method of asking for post mortems and their frequency. There is a far higher proportion of cases of undiagnosed and rare disease in the post mortem series than in the total hospital deaths.

Age distribution of patients (Fig. 6)

As in most reported post mortem series, the patients were considerably older than in clinical series.

The clinical course of untreated lung cancer is usually only a matter of months, so the different ages of the biopsy and post mortem series cannot be explained by the latter patients being in a more advanced stage of the disease.

This older age distribution, like the sex distribution, is probably due to social and economic factors.

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Duration of stay in hospital

In the 107 cases where the time spent in hospital was known from the records, 32 per cent were dead within a week of admission and 81 per cent were dead within a month. This is similar to the cases reported by Budinger (1958) where 20 per cent were dead within a week of admission and 80 per cent died within three weeks of admission.

The biopsy series has shown that among 419 untreated and mainly inoperable cases of lung cancer only 22 per cent died within a month. Comparison of the survival rates of these two series suggests that very few hospital beds can have been used as " chronic sick " beds, and that the cases in the post mortem series were very different clinically from the inoperable cases in the biopsy series.

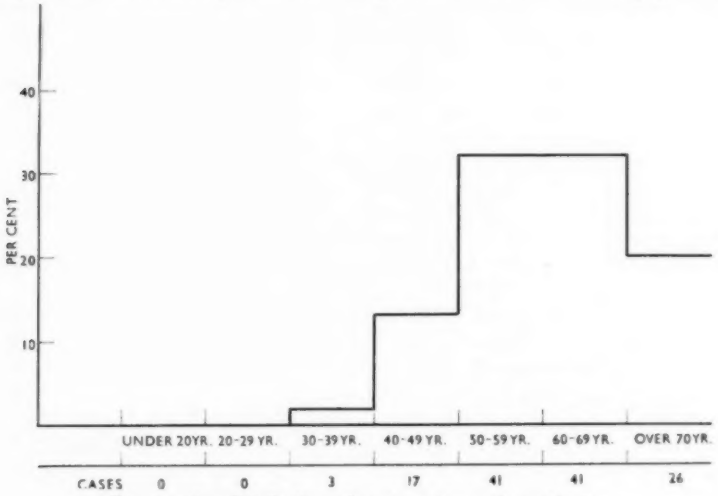

FIG. 6.—Age distribution of post mortem series patients..

Symptoms causing admission to hospital (Table VI)

In only 30 per cent of cases were the symptoms which caused the admission of the patients referable to the respiratory system. In 43 per cent of cases the main symptoms were caused by distant metastases.

TABLE VI.—*Systems Giving Rise to Symptoms which lead to Admission of Post Mortem Series Patients*

Medical		
Respiratory system (primary lesions)	48	
Central nervous system (cerebral metastases cerebral thrombosis, peripheral neuropathies)	14	
Cardiovascular system (coronory thrombosis, pericardial involvement)	8	86
Haematological (thrombocytopenia, leucoerythroblastic anaemia)	5	
Vague (anorexia, asthenia, loss of weight etc.)	11	
Surgical		
Gastrointestinal tract (liver, pancreas and peritoneal metastases and peptic ulcers)	24	
Orthopaedic (fractures, rheumatism, osteodystrophy)	7	37
Urinary (renal secondaries, unrelated prostatism)	6	

In 30 per cent of cases these symptoms were mainly " surgical " and at the time of death 18 per cent were still in surgical wards. In eight cases symptoms and death were due to apparently unrelated diseases (cerebral thrombosis 1, coronary thrombosis 1, bleeding or perforation of peptic ulcers 5).

The surprisingly high mortality from peptic ulcers in this series does not represent the whole incidence of peptic ulceration among the cases, as many ulcers must have been overlooked. A similar association was noted by Lea (1952) who found that 12·1 per cent of a necropsy series of lung cancer cases had peptic ulceration.

Diagnostic accuracy

The accuracy of clinical diagnosis has been assessed—from case records in 111 cases, and from post mortem report clinical abstracts in 12 cases (where the case records were missing at the time of review). Five cases who were brought to hospital dead or died in the Casualty Department have not been included. Case records of patients whom I thought had not been correctly diagnosed clinically have been assessed independently by a consultant physician to the hospital, who has always agreed with my own opinion.

A correct clinical diagnosis was made with 48 per cent of patients. No mention of lung cancer appears in 25 per cent of the case records. The remaining 27 per cent were difficult to assess — usually secondary carcinoma was diagnosed and the primary site was uncertain though the lung was considered a likely site. It is clear from the case notes that once a firm diagnosis of secondary malignant disease had been established the clinicians desisted from further investigations for humanitarian reasons.

Diagnostic accuracy was lower in women (28 per cent) than in men (52 per cent). To a considerable extent this was because the possibility of lung cancer is often overlooked with female patients, but also it was due to the higher proportion of women suffering from metastatic disease, which itself was due to the cell-type distribution in women.

Three cases were diagnosed wrongly at post mortem examination as pancreatic carcinomas, and the diagnoses were corrected after histological examination and re-examination of the lungs. All three had oat-cell carcinomas.

The diagnostic accuracy of this series (48 per cent) is considerably lower than the 61 per cent in cases reported by Willis (1960) and the 54 per cent reported by Bonser (1959). The explanation is probably that the post mortem rate at Broadgreen Hospital was 17 per cent lower than in Willis' series, and naturally included a higher proportion of undiagnosed cases. If it is assumed that hospital deaths registered as due to lung cancer but without post mortem examination were correctly diagnosed, the diagnostic accuracy in the ten year period would be 77 per cent. This figure is probably close to the truth in a hospital which by the proximity of the Thoracic Centre is unusually conscious of lung cancer.

Histological Features (Table VII)

Specificity of cell-types

Blocks of tumour from the primary site, regional lymph nodes, local spread and distant metastases were examined. 513 blocks of tumour were taken from 128

cases, an average of five blocks in all cases where the growth had extended beyond the primary site. All sections have been re-examined.

In 119 cases the cell-type remained constant throughout the tumour and its metastases. In nine cases (7 per cent) variations in cell-type were seen, and usually when these were present, mixtures of at least three cell-types were found.

Cell-type characteristics

Squamous carcinoma occurred infrequently, but these cases were the most accurately diagnosed cancers in the series. Only a third of the patients showed any metastic tumours and death was usually due to septic lung complications or unrelated conditions.

Oat-cell carcinoma was the commonest form of tumour with the highest incidence of metastases and, probably for this reason, was the least accurately diagnosed.

Carcinoma simplex was slightly less common than squamous carcinoma, but was diagnosed correctly almost as often. Metastases were more frequently seen than were the squamous carcinomas, but less so than in the other types.

Adenocarcinoma was the second commonest tumour, and a high proportion of them had metastases. Diagnostic accuracy with these tumours was low.

MASS X-RAY CAMPAIGN, LIVERPOOL 1959

In February and March 1959 a Mass X-ray Campaign was held in Liverpool, in which 454,286 people were X-rayed, representing 76·5 per cent of the population over the age of 15 years. The surgical aspects of this campaign have been reported by Waddington (1960).

118 proved cases of lung cancer were discovered, and in 101 cases the histological type of tumour was reported from biopsy or resection specimens.

As such a campaign provides another cross-section of lung cancer patients all available tumours have been re-examined and re-typed where necessary. Ninety-two cases were diagnosed or operated on in Broadgreen Hospital or Aintree Hospital and all these sections were available. There is, therefore, some overlap of this series with the biopsy and operation series. Dr. E. Mavis McConnell of Liverpool Chest Hospital and Dr. H. Vickers of Walton Hospital each gave biopsy specimens from cases where no resection was undertaken.

The cell-type distribution of these 95 cases is shown in Table VII.

TABLE VII.—*MMR. Survey—Liverpool* 1959

Squamous carcinoma	40	42 per cent
Oat-cell carcinoma	16	16·5 per cent
Carcinoma simplex	17	17·5 per cent
Adenocarcinoma	14	14·5 per cent
Adenomatosis	1	1 per cent
Carcinoma	7	7 per cent

DISCUSSION

This study was undertaken in an attempt to find answers to four questions, which were :—Is lung cancer so pleomorphic that histological classification is valueless ? If not, do the cell-types of lung cancer have characteristic biological

properties ? What is the cause of the differing cell-type frequencies reported in many series ? What is the true cell-type distribution of lung cancer in a population ? It is thought that satisfactory answers can now be given to the first three questions, but that there is no practical way of finding the answer to the last question.

Apart from these problems, the study has produced evidence which calls for modification of an accepted method of assessing histological alterations in the incidence of lung cancer in a population (Kreyberg, 1959), and other evidence which conflicts with arguments still used in support of the view that there may not be any increase in the incidence of lung cancer (Willis 1960). Both these points will be discussed.

Mixed-cell Types

Their frequency in the bronchial biopsy carcinomas was only 1·5 per cent, but because of the small amount of tissue available in each case little can be deduced from this series. In the operation specimens the incidence of mixed types was 3 per cent, and as an average of four blocks of tumours was examined in each case, as well as any lymph nodes attached to the specimens, this figure is of considerable value. The highest frequency of mixed types, 5·5 per cent, was found in the post mortem cases, in which an average of five tumour blocks was examined in cases where metastases were present, the blocks including tissues from the metastases.

A higher proportion of mixed types was found in cases where more blocks were examined, but I do not think that this process could be extended further, or that if ten blocks per case has been studied the incidence of mixed types would have become very much higher.

Half the mixed tumours contained mixtures of all cell-types, and often these mixed patterns were found in all sites and metastases examined. The other half of the mixed tumours were roughly equal numbers of squam-adenocarcinomas, squam-oat-cell carcinomas and adeno-oat-cell carcinomas.

If squamous metaplasia on the surfaces of oat-cell tumours and adenocarcinomas had been classified as mixed tumours, and if a glandular pattern in parts of oat-cell carcinomas had been called adeno-oat-cell carcinoma, the frequency of mixed cell-types might have been about 20 per cent of lung cancers, which is near the 23 per cent quoted by Willis (1960). However, I agree with Walter and Pryce (1955) that these features do not represent mixed forms of lung cancer.

Biological Features of the Cell-types

Even though consistency of cell-type has been found in lung cancer it does not follow that cell typing is of value unless it can be shown that individual cell-types have characteristic biological and prognostic qualities.

Many attempts have been made at grading other malignant tumours, mainly based upon degrees of differentiation and rate of cell division, but in lung cancer the problem is more difficult as it involves assessing the significance of several quite different histological pictures and attempting to show the inter-relationship of tumours of diverse appearance. Many arrangements of the cell-types, or spectra

of lung cancer, have been made, based upon different factors such as histological similarity, supposed site of origin, relative malignancy or theories of aetiology.

In the present study the existence of mixed cell-type tumours has shown that the final entity is lung cancer; but also it has been possible to arrange the cell-types in an order or gradation according to the features, which have been described in this survey and are summarised in Table VIII. This order is—squamous carcinoma, carcinoma simplex, adenocarcinoma, oat-cell carcinoma; and the biological features which fit into this arrangement are the sex ratio, age distribution, size of tumour and regional lymph node involvement, and frequency of metastases.

TABLE VIII.—*Cell Type Characteristics from all Series of Lung Cancer*

	Squamous carcinoma	Carcinoma simplex	Adeno-carcinoma	Oat-cell carcinoma
SEX—m/f ratio in biopsy, operation specimen and post mortem specimen series	22:1, 23:1, 17:1	17:1, 14:1, 6:1	5·9:1, 5·4:1, 3·3:1	4·1:1, 3·3:1, 3:1
AGE—percentage under 50 years age at time of diagnosis	17 (male) 21 (female)	17	20	35 (male) 43 (female)
SIZE OF PRIMARY TUMOUR—percentage of tumours in operation series under 40 mm. diameter	67	60	58	55
LYMPHATIC SPREAD—percentage of large tumours in operation series with lymph nodes involved	35	42	50	66
BLOOD-BORNE METASTASES—percentage in post mortem series	33	60	65	81
SURVIVAL—percentage alive in biopsy series cases three months and six months after diagnosis	56 35	—	—	38 12
DIAGNOSTIC ACCURACY—percentage correctly diagnosed in post mortem series	64	60	41	35

The table shows that carcinoma simplex has features which are very similar to those of squamous carcinoma and that in many ways oat-cell carcinoma is similar to adenocarcinoma, particularly regarding sex distribution, lymphatic spread and blood-borne metastases. This pairing of squamous carcinoma with carcinoma simplex, and adenocarcinoma with oat-cell carcinoma has not been based upon any histological similarity but solely on the other findings of the survey.

Histologically it is easy to accept that there could be a close link between squamous carcinoma and carcinoma simplex by assuming that the latter are all poorly differentiated squamous tumours. Both types usually arise from the larger bronchi. It is not so easy to accept a link between adenocarcinoma and oat-cell carcinoma, in spite of the acinar structure of some oat-cell tumours. Oat-cell tumours are usually central growths and many have a demonstrable bronchial site of origin, while adenocarcinomas are peripherally placed and detailed examination nearly always fails to show any bronchial origin. The nuclei of these two types of tumour are usually quite different. Another interesting but little recorded feature of oat-cell tumours is the occurrence of pools and strands of haemotoxophilic Feulgen-positive material in necrotic parts of many tumours, particularly in the walls of small vessels, which has been described by Assopardi (1959). This curious phenomenon was seen in many oat-cell carcinomas in the present study, but never in adenocarcinoma or in any other form of lung cancer.

Explanation of Variable Cell-type Frequencies in Different Series

This general question can be answered by referring to the present series, which are typical of many other which have been recorded.

Biopsy series

The bronchial biopsy carcinomas reflect the true cell-type distribution of cancers which arise in the larger bronchi, are within reach of the bronchoscope and usually produce early symptoms. Squamous carcinomas were the commonest types but oat-cell tumours were present in a third of the cases, while adenocarcinomas were rarely found.

The only selection in this series arose from the site of the tumour and perhaps the age of the patients, because if bronchoscopies had been performed on older patients probably the frequency of squamous carcinoma would have been even higher.

There are few accounts of purely bronchial biopsy lung cancers for comparison with the present cases.

Operation specimen series

Though these cases are gathered in a wider diagnostic net than the biopsy series, they do not represent the true cell-type distribution of lung cancer. Selection of cases for operation is naturally influenced by the size of tumour, the degree of local spread and the presence of metastases. Choice of cases suitable for surgery is also influenced by the attitude of surgeons to different cell-types of cancer, which often are known before operation is decided upon.

As squamous carcinoma remains localised longer than do other forms, and as some surgeons are prepared to attempt radical surgery on borderline carcinomas if they are squamous tumours but not if they are oat-cell carcinomas, the frequency of squamous tumours is always high and that of oat-cell tumours low in any operation series compared with a biopsy series, and the cell-type frequencies are further altered by the inclusion of peripheral adenocarcinomas.

Post mortem series

This is a highly selected series and though by many considered to be representative of the overall cell-type distribution I regard post mortem studies as the least reliable method of assessing the frequency of lung cancer, or of its cell-types, in a population.

As most acute general hospitals try not to keep in their wards cases of diagnosed inoperable lung cancer these victims mostly die in " chronic sick " hospitals or in their homes. When diagnosed cases die in hospital they frequently escape post mortem examination because, the diagnosis being known and the condition common, they are not of much general interest. For these reasons the post mortem series contains a high proportion of patients who died shortly after admission (before there was time for diagnosis or transfer elsewhere), of patients who died from acute complications of lung cancer (such as pneumonia or pericarditis) and of patients who had symptoms solely from metastatic tumours (often with unknown primaries). Other ways in which the series is not typical of lung cancer are the sex and age distribution.

As squamous tumours are relatively easily diagnosed, produce symptoms early and metastasise late, they were uncommon in the post mortem series. When they occurred, admission had usually been due to acute complications such as pneumonia, or unrelated conditions like the complications of peptic ulceration.

On the other hand, oat-cell carcinoma and adenocarcinoma were common, as they were more difficult to diagnose, are relatively common in women, and often metastasise early and produce their main symptoms from the metastases.

Mass radiography series

This was a mixed series comprised of both biopsy and operation specimens with the latter providing the histological diagnosis in 68 per cent of cases. In the present series, as in most others, compared with an operation series, the frequency of squamous carcinoma was low, probably because patients with squamous carcinoma had had earlier symptoms and had attended hospital clinics rather than mass radiography centres. The incidence of adenocarcinoma was higher than in the operation series.

The observations based upon the present series appear to be true of most published studies. By looking at the cell-type frequencies in recorded series it is nearly always possible to anticipate the type of material that has been studied.

The difference in cell-type frequencies found in lung cancer collected from different types of material is so great that from an epidemiological angle it is useless to report a series where the source of the material is not precisely defined, and it is only of limited value to report cases from mixed sources unless the cases from each source are analysed separately.

Cell Typing as an Indicator of Lung Cancer Frequency

The increased incidence of lung cancer over the last half century is mainly attributed to cigarette smoking and atmospheric pollution. In 1954 Kreyberg divided lung cancers into two groups which he thought were aetiologically distinct. His Group I contained squamous, large cell and small cell carcinoma, which he considered to be caused by external factors such as cigarette smoking and atmospheric pollution. Adenocarcinomas, adenomatosis and bronchial adenomas made up his Group II, which he found were more equally distributed between the sexes and thought were unrelated to external factors.

This view was supported by Doll, Hill and Kreyberg in 1957, when they showed that there is a close relationship between the amount of tobacco smoked and the development of squamous, large cell, and small cell carcinoma but no such correlation existed with the Group II tumours. In 1959 Kreyberg omitted large cell carcinoma from his Group I tumours, but showed that these tumours are also related to other external factors, such as nickel and asbestos. He put forward the thesis that the ratio between Group I and Group II tumours in men in any consecutive unselected material provides information as to the frequency of lung cancer in a population, and that an alteration in this ratio will indicate a change in the lung cancer situation. In support of this theory he quoted Norwegian figures, where the ratio before the increase of lung cancer was 1·8 : 1, and later became 38 : 1. However, a later study (Ferrari and Kreyberg, 1960) of 78 cases in Venice,

where lung cancer is prevalent, showed a ratio of 3·2 : 1, not much different from Norway where lung cancer has a low incidence.

I think that Kreyberg's basic conception of a division of lung cancer into two aetiologically distinct groups is an approach that may be of great value, and probably carcinoma simplex (his large cell carcinoma) should appear in Group I, as it behaves so like squamous carcinoma. However, his method of comparing group ratios in different series and deducing therefrom lung cancer incidence changes needs closer examination and some standardisation of studied series is essential.

The present survey has shown that the cell-type frequencies and, therefore, the Group I : II ratio, varies according to the type of material being studied. Liverpool is certainly a city with a very high incidence of lung cancer, but the Group I : II ratio in men (confining Group I to squamous and oat-cell tumours) was 17·8 : 1 in the biopsy series, 5·04 : 1 in the operation specimens and only 1·84 : 1 in the post mortem cases. These three ratios were obtained from studies of the same population over a constant period, and according to Kreyberg's theory show that in the same period Liverpool had about three times as much, and only two thirds of, the lung cancer incidence of Norway.

It is obvious that all bronchial biopsy series will have a very high ratio, for example, the cases typed by Doll, Hill and Kreyberg (1957), which one can deduce were mainly diagnosed from bronchial biopsies, have a ratio of 20·8 : 1. All post mortem series will have a low ratio because of the high proportion of adenocarcinomas and low proportion of squamous tumours; and operation series will occupy an intermediate position.

In his 1959 paper Kreyberg illustrates the value of using his ratio for assessing increases in lung cancer by comparing Norweigian figures up to 1946 with those after 1948, which showed that the ratio had doubled. Unfortunately the value of this example is reduced by the knowledge that the earlier ratio was obtained from post mortem material, while the later ratio was from mainly clinical material. In spite of these obvious defects which may have lead to a false conclusion, I think that a study of the Group I : II ratio in large and strictly comparable materials may provide an accurate index of any increase of lung cancer caused by cigarette smoking or atmospheric pollution.

The Increase in Lung Cancer

One other aspect of lung cancer deserving comment is its increased incidence in recent years. In the ten year period of this study the number of cases of lung cancer registered with the Liverpool Cancer Control Organisation more than doubled, the number of positive carcinoma bronchial biopsies nearly doubled (allowing for those seen at new clinics), the number of operation specimens rose steeply and steadily, and there was a small increase in the post mortem material.

In spite of this clinically-apparent increase there are still those who claim that there is as yet no proof of an increase in lung cancer, but that an apparent increase may be due to better diagnosis, unproved diagnosis, or an ageing population. One of the common arguments used against there being much increase is based upon post mortem statistics (Willis, 1960).

Though an increase has been apparent clinically, and in the Registrar General's returns since the first World War, the percentage of lung cancer cases in post

mortem series up to 1930 remained constant. Since then there has been an increase of about 50 per cent in the post mortem figures. Willis points out that in the period when there was a ninefold increase in death certificates from lung cancer the increase in the post mortem rate was only 50 per cent.

Nearly all these statistical studies have been made in teaching hospitals, where the post mortem rate is not always very high and where there are few cases of diagnosed inoperable malignant disease. The two factors which mainly influenced the frequency of lung cancer in the post mortem series at Broadgreen Hospital were the transfer of cases to hospitals for the chronic sick and the post mortem rate. The same factors would influence a similar series in a teaching hospital where, though the post mortem rate might be higher than at Broadgreen Hospital, there would be non-admission of cases known to be inoperable, and more transfer of cases found to be inoperable. Also as post mortem series which are not 100 per cent of deaths contain a higher proportion of undiagnosed cases than do the hospital deaths, and as bronchoscopy is now a common investigation making lung cancer more easily diagnosable, a smaller proportion of lung cancer cases now occurs in nearly all post mortem series. These factors clearly affected the lung cancer incidence in the present post mortem series, but they are often overlooked by those who study post mortem statistics and draw conclusions based purely on mathematics.

SUMMARY

An analysis of the histopathology of over 1900 cases of lung cancer occurring in the Liverpool area in the decade 1950–1960, showed that in no more than 6 per cent of cases was a mixture of cell-types found in the tumours. The rest of the lung cancers were classified as squamous carcinomas, carcinoma simplex, oat-cell carcinoma, adenocarcinoma and adenomatosis.

A study of the clinical records at a follow-up of patients showed that cell-types possessed distinctive biological properties, regarding age and sex distribution, size of tumours, lymphatic spread extent of metastases and survival. In order of increasing malignancy the tumours were: squamous carcinoma, carcinoma simplex, adenocarcinoma and oat-cell carcinoma.

The lung cancers were studied in three main series, in which the histological diagnosis had been made from specimens obtained at biopsises, operation and post mortem. The cell-type frequencies found in the series showed marked differences, which could be explained as being due to the biological properties of the cell-types and to external factors influencing the selection of cases in each series. None of the series reflects the cell-type frequency in a population.

The method of using the Group I tumour : Group II tumour ratio as in indication of the frequency of lung cancer in a population is only of value if strictly comparable series are studied.

I wish to thank the Medical Staffs of the Regional Thoracic Service and of Broadgreen Hospital, for the use of their patients' case records; Dr. L. Findlay for his assessment of clinical diagnostic accuracy of the post mortem series patients; Dr. E. Mavis McConnell, Pathologist to the Liverpool Cancer Control Organisation, for much assistance in tracing patients' records; and Dr. Rachel M. Rawcliffe for reading and correcting the manuscript.

Especially do I wish to acknowledge my debt to Mr. A. Robertson, F.I.M.L.T., Senior Histology Technician, not only for all the histological preparations which I have examined, but also for his tireless assistance in finding old sections and in abstracting and tabulating old reports, without which help the work could not have been possible.

REFERENCES

ANDERSON, W. A. D.—(1948) 'Pathology'. London (Henry Kimpton), p. 745.
AZZOPARDI, J. G.—(1959) *J. Path. Bact.*, **78**, 513.
BARNARD, W. G.—(1926) *Ibid.*, **29**, 241.
BIGNALL, J. R.—(1958) In 'Carcinoma of the Lung'. Ed. Bignal, J. R. London (Livingstone), pp. 168, 184, 185.
Idem AND MOON, A. J.—(1955) *Thorax*, **10**, 183.
BONSER, G. M.—(1959) *Brit. J. Cancer*, **13**, 1.
BUDINGER, J. M.—(1958) *Cancer*, **11**, 106.
CHRISTIANSEN, T.—(1953) *Brit. J. Cancer*, **7**, 428.
DOLL, R. AND HILL, A. B.—(1952) *Brit. med. J.*, ii, 1271.
Iidem AND KREYBERG, L.—(1957) *Brit. J. Cancer*, **11**, 43.
FERRARI, E. AND KREYBERG, L.—(1960) *Ibid.*, **14**, 609.
GALLUZZI, S. AND PAYNE, P. M. (1955) *Ibid.*, **9**, 511.
GIFFORD, J. H. AND WADDINGTON, J. K. B.—(1957) *Brit. med. J.*, i, 723.
HINSON, K. F. W.—(1958) in 'Carcinoma of the Lung'. Ed. Bignall, J. R. London (Livingstone), pp. 115, 133.
JAKOBSEN, A.—(1953) *Brit. J. Cancer*, **7**, 423.
KREYBERG, L.—(1954) *Ibid.*, **8**, 199.—(1959) *Acta. Un. int. Cancr.*, **15**, 78.
LEA, A. J.—(1952) *Thorax*, **7**, 305.
LIEBOW, A. A.—(1952) 'Tumours of the Lower Respiratory Tract. Atlas of Tumour Pathology'. Sec. 5. Fasc. 17. Washington, D.C. (Armed Forces Institute of Pathology), p. 67.
MCBURNEY, R. P., MCDONALD, J. R. AND CLAGETT, O. T.—(1951) *J. thorac. Surg.*, **22**, 63.
MASON, G. A.—(1949) *Lancet*, ii, 587.—(1960) *Thorax*, **15**, 1.
MULLANEY, P. J.—(1958) *Brit. J. Cancer*, **12**, 327.
NICHOLSON, F., FOX, M. AND GRAHAM BRYCE, A.—(1957) *Lancet*, i, 296.
PHILLIPS, F. J., BASINGER, C. E. AND ADAMS, W. E.—(1950) *J. thorac. Surg.*, **19**, 680.
RAEBURN, C. AND WALTER, J. B.—(1956) *Lancet*, i, 778.
SELLORS, T. H.—(1955) *Brit. med. J.*, i, 445.
STEINER, P. E.—(1953) *Acta Un. int. Cancr.*, **9**, 42.
UMIKER, W. AND FRENCH, A. J.—(1960) *Cancer*, **13**, 1053.
WADDINGTON, J. K. B.—(1960) *Med. Offr*, **104**, 293.
WALTER, J. B. AND PRYCE, D. M.—(1955) *Thorax*, **10**, 107, 117.
WHITWELL, F.—(1961) *Brit. J. Cancer*, **15**, 429.
WILLIS, R. A.—(1960) 'Pathology of Tumours'. 3rd edition. London (Butterworth), pp. 130, 77, 72, 360, 369.

UMI

X-RAY SIEVE THERAPY OF BRONCHIAL CARCINOMA*

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A PRESSING medical question, which is as yet unanswered, is that of the role of radiotherapy in the management of inoperable cancer of the lung. That opinions regarding the beneficial effects of radiotherapy differ widely is confirmed by the varying approach to treatment in different centres. Advances in thoracic surgery have brought in their wake a considerable deterioration of the type of case referred for radiotherapy. In addition, some radiotherapy centres do not accept cases of carcinoma of the lung for treatment unless they present with signs of superior vena caval syndrome, haemoptysis, dysphagia or pain.

The initial policy of both thoracic surgeons and physicians is often either " surgery or nothing ", and radiotherapy is used only when patients are in extremis, requiring some symptomatic relief. Frequently chemotherapy is given in preference to radiotherapy, even in cases belonging to histological groups not sensitive to chemotherapy. With the advent of more powerful machines the question of the usefulness of radiotherapy in bronchial carcinoma has been reopened, as is shown by several controlled trials which are under way. Whatever the terms of these trials, it is desirable to assess the value of several radiotherapy techniques at 200–250 kV, and not only those that are conventional. The present paper on the results of treatment with X-ray sieve therapy (Jolles, 1949, 1952, 1953, 1960) claims that this approach is better than more conventional methods at 200–250 kV. This is of importance if future claims of supervoltage therapy are to be assessed fairly, when the results obtained with lower voltage techniques should be available.

The survival rates after radiotherapy throw some light on the advisability of so-called radical radiotherapy, but the clinical management of patients with this dreaded disease suggests that the question of how the patient lives before death overtakes him is a most important one. In the assessment of results it is the symptomatic improvement of the patient treated by radiotherapy that indicates any benefit received.

Indications for sieve therapy

Two common findings indicate the need for the adoption of sieve therapy.

(1) The site. The vicinity of vital organs apart from those vital structures already involved in the neoplastic process, and the liability of lung tissues to radiation fibrosis and secondary infection indicate that conventional methods of treatment are beset with great difficulties. If radiation effects on normal tissues are to be diminished new approaches must be tried.

* Based on an invited paper at the IX International Congress of Radiology, Munich, July 1959.

(2) The extent of the disease. In about 80 per cent of cases, wide involvement of the regional lymph nodes is found at the commencement of treatment. Volumes with a long axis not less than 12 to 15 cm. usually have to be included in the irradiation field. In the majority of patients the treatment of small volumes is futile. After a thoracotomy has been performed, the volume to be treated is still larger, emphasising the need for large field therapy.

METHOD

The sieve or grid method satisfies these two requirements. Fractionation of radiation in space as well as in time allows the delivery of large amounts of radiation in depth without overloading the skin.

The principle of the method is that, with a lead chess-board sieve, portions of the tumour surface, 0·5–1·0 cm. square, are exposed to radiation while other portions of similar area are protected, so that important structures remain undamaged and can take part in repair. For superficial tumours, and on occasion for deep seated tumours, the position of the radio-opaque and transparent areas is reversed and a second course of treatment is given, to the previously unexposed areas. The already treated areas are thus protected and their recovery is not hindered.

Sieves with an open to protected area 50 : 50 ratio were used in the Northampton series, while sieves with 50 : 50, 40 : 60 and 34 : 60 were applied in cases reported from other centres (v.q.). In a few cases sieves of 34 : 60 ratio were used while approximately two-thirds were treated with 40 : 60 sieves. The ease with which complementary sieves can be used in subsequent series of treatment, makes the 50 : 50 sieves, though not so easy to manufacture, on the whole preferable.

The sieve openings were 0·8–1·0 cm. in diameter. The kilovoltage was 200–250, and the H.V.L. in the great majority of cases was 2 mm. Cu. The overall treatment time varied from 4–6 weeks, but in some cases after an interval of 6–8 weeks a second series of treatments was given.

The daily dose varied between 500–800 r and on occasions 1000 r on either one or two fields were given throughout the treatment series.

The depth dose was assessed as " average " or the mean of the " maximum ", i.e. of the dose under open areas and the " minimum " in the shaded portions. Mean tumour doses ranging from 2500 r to 4500 r were given in the Northampton series.

RESULTS

1976 cases of carcinoma of the lung were registered in the Oxford Regional Hospital Board Area during the years 1954–1957 inclusive. This Region has a population of 1·5 million and is served by three radiotherapy departments situated in Northampton, Oxford and Reading. In the period under review, 441 cases of carcinoma of the lung were treated with radiation to the primary, 249 with surgery, 100 with various forms of treatment such as nitrogen mustard or combined surgery and radiotherapy, while 1186 received no treatment whatsoever. The majority of the latter cases was never referred for radiotherapy and some had an exploratory thoracotomy. All patients treated by the sieve method were selected only in that the disease was always very advanced.

UMI

Of the 441 cases treated with radiation 140 were dealt with at Northampton (117 sieve, 23 conventional), 212 at Oxford (134 conventional and 78 sieve or combined sieve plus conventional) and 89 at Reading (conventional).

The general condition of the 23 patients treated with conventional techniques in the Northampton series was better and their disease was considered less advanced than that of those treated with the sieve technique.

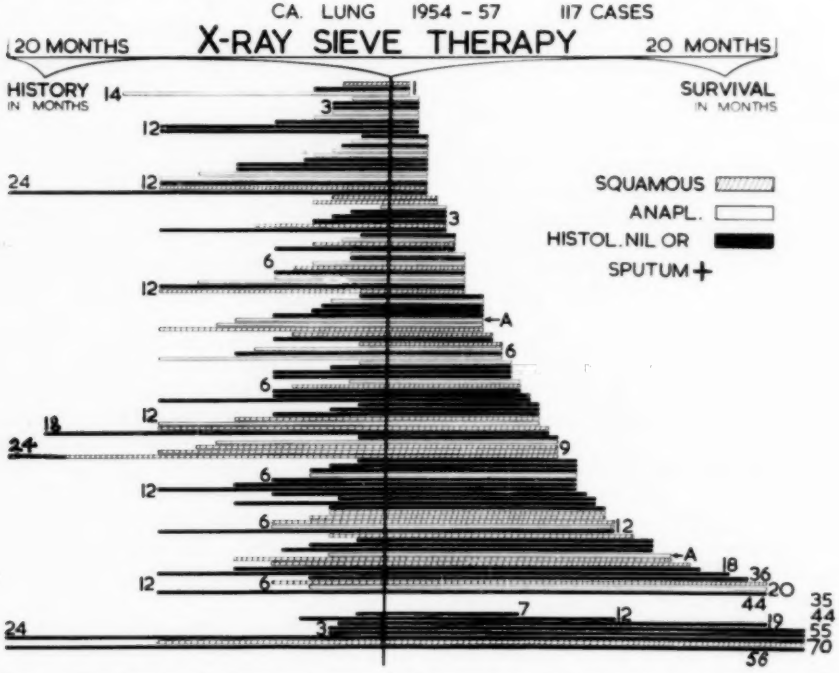

FIG. 1.—The length of history and survival of patients treated with the sieve technique at Northampton.

In Fig. 1 are shown the length of history and survival in 117 cases treated with the sieve technique at Northampton. Nine cases which did not complete treatment are included. "A" denotes cases with adenocarcinoma. The bottom 8 cases were alive when the histogram was drawn.

A symptomatic improvement was noted in almost all cases from the beginning of treatment, which was very well tolerated. The skin reaction was not brisk; a mosaic of small areas of moist disepithelisation, which healed within two weeks leaving a pigmentation, was all that was noted on the anterior chest field after doses from 10,000–12,500 r over a period of 4–5 weeks. Only dry desquamation and a few oozing spots were produced by 9,000 r on the patients' backs. This, in patients with an A.P. diameter of 21 cm., gave at 2·25 mm. Cu. H.V.L. a maximum depth dose of 4600 r, minimum 2600 r, average 3600 r. When an exploratory

thoracotomy had been performed previously a third sieve field was added on the lateral chest wall (5000–6000 r). Postradiation fibrosis of the lung was noted in only two cases.

The subdivision of the cases into histological groups in the 117 Northampton series (Fig. 2) and of 287 cases from another centre has revealed that the survival of the group of squamous cell carcinoma is slightly longer than in the other groups. In a further subdivision of a series of 404 cases from 2 centres into the " over "

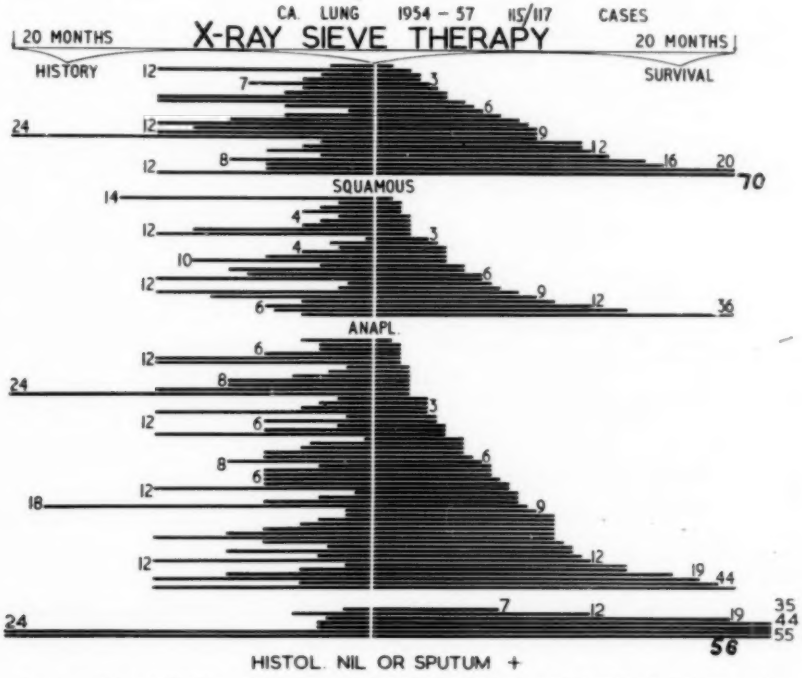

FIG. 2.—The subdivision of the cases into histological groups in the Northampton series.

and " under " 60 years of age groups, the former appeared to be doing slightly better and the length of history before treatment has been greater in the over 60 group ; this was also found in the German series of cases.

In Table I are shown the survival times of the cases treated with the two techniques.

In Table II are set out the survival times of patients who lived for more than 3 years after treatment.

Generally it is not the length of survival that matters but the quality of the improvement that is brought about by the treatment. With this in view it was thought that it might be useful to evaluate the clinical results using the criteria adopted by Karnofsky *et al.* (1951) and Mitchell (1960), in their respective studies of the results of treatment with nitrogen mustard, and the role of Synkavit as a

TABLE I.—*Oxford Regional Board Area, Carcinoma of the Lung 1954–1957 Inclusive*

Method of treatment	Number of cases	(September 1, 1960) Alive at : 3 months	6	12	24	36
Radiotherapy :						
Sieve : Northampton	117	89 (76%)	61 (52%)	21 (18%)	7 (6%)	6 (5%)
Sieve : all cases	195	138 (71%)	102 (52%)	44 (23%)	8 (4%)	6 (3%)
Conventional fields	246	134 (55%)	73 (30%)	22 (9%)	8 (3%)	2 (0·8%)
Surgery*	249	—	—	—	—	—
Various	100	59 (59%)	40 (40%)	21 (21%)	8 (8%)	2 (2%)
No treatment	1186	450 (38%)	230 (19·5%)	105 (9%)	22 (2%)	4 (0·34%)

* Follow-up data not yet complete.

TABLE II.—*The Survival Times of Patients who Lived for More than 3 Years after Treatment*

Sieve fields			Conventional fields		
Survival in months	Histology	Dose average	Survival in months	Histology	Dose average
		Alive			
36	? pos. +	High dose	36	Anaplastic	High dose
44	Sputum pos. +	3000 r	36	Squamous cell ca.	" "
56	Nil	3600 r	36	? pos. +	" "
70*	Squamous cell ca.	3600 r	36	Nil	—
			72	Squamous cell ca.	6000 r
		Dead			
36	Nil	3000 r			
36	? pos. +	High dose			
40	Squamous cell ca.	" "	58	Nil	High dose
43	Nil	" "			
44	Sputum pos. +	2875 r			
56	" " "	4375 r			

* This patient who received sieve treatment from 21/12/54 to 21/1/55 for a poorly differentiated squamous cell carcinoma in the right upper lobe bronchus, developed 68 months later, another tumour of the same histological pattern in the left lung, for which he was also treated with sieve therapy. He was alive and fairly well at the end of April, 1961.

sensitizer in radiotherapy of bronchial carcinoma. The subjective and objective improvement shown by the relief of symptoms and measurable regression of lesions (X-ray films, etc.) set out in the " Performance Status " which attempts to measure the patients' ability to continue useful work, etc., was studied in the Northampton cases. The period of improvement and relative well-being represented approximately 75 per cent of the survival time after treatment.

In order to substantiate the conclusions regarding the role of sieve therapy in bronchial carcinoma a questionnaire was sent to several radiotherapy centres in the United Kingdom and abroad. Collaboration, for which the author is greatly indebted, was ensured from 8 centres (Professor Sir Brian Windeyer and Miss M. D. Snelling, Middlesex Hospital, London ; Professor R. Paterson, Christie Hospital, Manchester ; Dr. A. G. C. Taylor, Royal South Hants Hospital, Southampton ; Dr. P. Kröker, Evangel. Krankenhaus, Essen, and Dr. W. Pfeifer

and Dr. K. Seidel, Oldenburgisches Landeskrankenhaus, Sanderbusch in Germany, and of Professor H. Kaneda, Shinshu University, Kyoto, Japan). Including 14 cases reported by Hohl in Zurich (1953) and 110 cases by Haubrich and Thurn (1957) and the above discussed cases from Northampton, as well as 78 cases treated with the sieve or sieve plus conventional techniques by Dr. F. Ellis, Churchill Hospital, Oxford, a total of 942 cases of bronchial carcinomas treated with the sieve was collected. Of these, 60 cases comprising mostly patients with superior vena caval syndrome were treated with a single massive dose of 4–5000 r through a sieve. In the centre in which these patients were treated it was thought that this technique gave worthwhile palliation. A further 86 cases received sieve therapy after surgery, either post-operatively or for recurrences, and will be discussed later.

In the group of 677 British sieve cases the ratio of male to female was 10 : 1. There were 150 squamous celled carcinomas, 139 anaplastic tumours, 12 adenocarcinomas and 316 were classified as " no histology ". In the " no histology " group were included a few positive malignant tumours which were not typed, and also a number of sputum-positive cases.

In Table III the overall results of treatment with the sieve method (inclusive of the Northampton and Oxford cases) are compared with cases treated with conventional methods.

TABLE III.—*The Overall Results of Treatment with the Sieve Method Compared with Conventional Methods*

Method	Number of cases	Alive at 6 months	12 months	24 months	36 months
Sieve : Northampton	117	61 (52%)	21 (18%)	7 (6%)	6 (5%)
All centres	769	378 (49%)	158 (21%)	30 (4%)	11 (1·4%)
Conventional fields : Four centres	483	138 (28·5%)	46 (9·5%)	15 (3%)	6 (1·2%)

Fig. 3 shows the survival of patients who received X-ray sieve therapy after pneumonectomy, lobectomy and exploratory thoracotomy. The time interval between the operation and radiotherapy, and the survival after the radiotherapy are shown for each case. It can be seen that a good degree of palliation can be achieved by sieve therapy post-operatively.

DISCUSSION

The published results of the treatment of bronchial carcinomas with X-rays in the 200–250 kV range make very sober reading. Average survival of 4 months after treatment in a large series of cases have been recorded by many authors (Fulton, 1949 ; Schulz, 1957).

It can be seen from the foregoing that the sieve method often gave better results than conventional techniques. The objective and subjective clinical improvement, achieved at very little expense to the patient in terms of untoward radiation reaction, extra morbidity and post-radiation lung changes, shows clearly the superiority of a method based on fractionation in space as well as in time.

When it is realised that the series presented in this paper consisted of very advanced cases and that attempts at a " cure " by intensive methods of radio-

therapy was deemed worthwhile only in very few, then the conclusions drawn seems quite justified. The operability rate in bronchial carcinoma does not exceed 15 per cent of all cases and the great majority of cases referred for radiotherapy are well beyond the scope of any radical attempt at cure. It can be argued whether there is any justification for submitting patients with advanced carcinoma of the lung to treatments which will produce only small gains in terms of survival, at the expense of suffering in a great proportion of cases who are beyond practical radiotherapy.

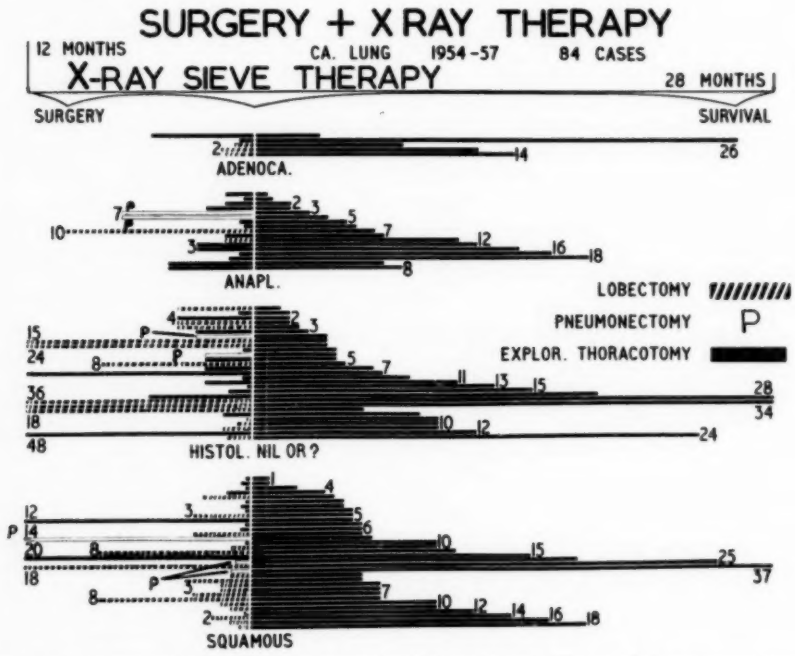

FIG. 3.—The survival of patients who received X-ray sieve therapy after pneumonectomy, lobectomy and exploratory thoracotomy.

The selection of cases for various types of treatment makes a comparison of published results of little value, and worthwhile conclusions cannot be drawn from statistical reviews unless these present the fate of all cases of bronchial carcinoma registered in the particular area. However, there should be no doubt that we must at all costs avoid causing by radiation any deterioration in the patient's condition. Treatment in selected cases with supervoltage machines does not produce much better results (Hohl, 1953; Haas, Harvey and Melchor, 1957). Morrison and Deeley (1960) reported a survival rate of 6 per cent at 3 years in a selected series of cases treated by an 8 MeV linear accelerator. These results do not yet justify any change in our decision as regards the main aims of treatment, either a radical attempt at cure, or palliation.

The claim that the sieve technique has an important place in the treatment of advanced bronchial carcinoma is well supported. It is also worthwhile stressing that with the sieve technique it is possible to deliver a high dose of radiation in depth over the large regions which have to be covered in most patients with bronchial carcinoma, without causing damage to lung and other tissues.

SUMMARY

A survey of 117 cases of bronchial carcinoma treated with the sieve method at Northampton has been made. The details of the technique are given and the results compared with those of two groups of cases treated in the Oxford Regional Board Area and various Departments throughout the world.

It is shown that the sieve technique has given better results than the conventional methods. Patients improve subjectively and objectively, without any untoward radiation effects. In addition, there are advantages of a biological and technical nature which indicate that sieve therapy has a role to play in the radiotherapy of bronchial carcinoma.

I wish to thank Dr. F. Ellis, of the Churchill Hospital, Oxford, and Dr. W. G. Evans, Royal Berkshire Hospital, Reading, for permission to make use of data concerning patients treated by them. I should like to acknowledge also the helpful co-operation of the chest physicians in Northants, Dr. O. E. Fisher, Dr. G. B. Lord and Dr. E. T. W. Starkie, and the thoracic surgeons Mr. C. Grimshaw, F.R.C.S. and Mr. G. C. Laurie Pile, F.R.C.S. of the Thoracic Surgery Unit, Churchill Hospital, Oxford.

I wish also to thank Miss C. Hunt of the Oxford Regional Records Bureau and Miss D. Smith of my department for help in assembling many of the data presented in this paper and Mr. G. B. Dun, M.S.R., for the illustrations.

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REFERENCES

Fulton, J. S.—(1949) *Proc. Roy. Soc. Med.*, **42**, 775.
Haas, L. L., Harvey, R. A. and Melchor, C. F.—(1957) *Cancer*, **10**, 154.
Haubrich, R. and Thurn, P.—(1957) *Strahlentherapie*, **102**, 180.
Hohl, K.—(1953) *Radiol. clin.*, **22**, 486.
Jolles, B.—(1949) *Lancet*, ii, 603.—(1952) *Brit. J. Radiol.*, **25**, 395.—(1953) 'X-Ray Sieve Therapy in Cancer'. London (H. K. Lewis & Co. Ltd.).—(1960) *Strahlentherapie*, Sonderband, **46** (II), 68.
Karnofsky, D. A., Burchenal, J. H., Armistead, G. C., Jr., Southam, C. M., Bernstein, J. L., Craver, L. F. and Rhoads, C. P.—(1951) *Arch. intern. Med.*, **87**, 477.
Mitchell, J. S.—(1960) 'Studies in Radiotherapeutics'. Oxford (Blackwell & Co.).
Morrison, R. and Deeley, T. J.—(1960) *Lancet*, ii, 618.
Schulz, M. D.—(1957) *Radiology*, **69**, 494.

BUSULPHAN IN THE TREATMENT OF CHRONIC GRANULOCYTIC LEUKAEMIA

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THE first chemotherapeutic agent used for the treatment of chronic granulocytic leukaemia was the heavy metal arsenic, which as early as 1865 was noted by Lissauer to exert a beneficial action in some cases. Radiotherapy as a treatment for this condition was first used in 1902 by Pusey and was found to be more predictable in action than arsenic. Over the next 50 years a wide variety of drugs was tried in the management of chronic granulocytic leukaemia but none was as satisfactory as radiotherapy which throughout this time remained the standard therapy. In 1953 Haddow and Timmis showed that the sulphonic acid ester busulphan (1–4 dimethanesulphonyloxybutane, Myleran, Burrows Wellcome & Co.) caused depression of granulopoiesis in man. Galton (1953) was the first to use this agent in the treatment of chronic granulocytic leukaemia and his results showed that this drug was capable of challenging radiotherapy as the treatment of choice.

Chronic granulocytic leukaemia is a relatively rare disease and although the large majority of untreated patients survive only three to four years, occasional patients may survive for eight to nine years (Minot, Buckman and Isaacs, 1924). Thus the final assessment of any new therapy requires considerable time. We have, over the past five years, treated 25 patients with chronic granulocytic leukaemia by busulphan and it is felt that a report of our experience may be of value. A preliminary report concerning five of these patients has previously been published (Nelson and Lowry, 1957). In addition, five of the patients in the present series have shown a very high platelet count during the course of their illness and it is of special interest that in four this occurred whilst they were on continuous busulphan therapy.

We have used busulphan in initial dosage of 4–6 mg. daily; subsequent control of dosage was as previously described by Nelson and Lowry (1957). Throughout the period of initial therapy all patients were hospitalized and given a high protein diet with a high fluid intake; blood transfusions were given only as supportive therapy. After discharge the patients were reviewed at least once a month as out-patients, and by the use of continuous busulphan therapy it was attempted to keep the white cell count in the range 10–20,000/cu.mm. but preferably below 12,000/cu.mm.

RESULTS

The results of busulphan therapy are best considered under two separate headings:

(*a*) Patients in whom it was the initial therapy.
(*b*) Patients in whom it was given following radiation or other chemotherapy.

Group (a)

This group of 16 patients who all presented with the typical haematological picture of chronic granulocytic leukaemia, contained seven males and nine females, varying in age from 24 to 67 years. Fourteen of these patients were treated by us throughout and two (cases 12 and 16) were referred after therapy elsewhere. Twelve of the 14 patients given busulphan initially by us achieved complete clinical and haematological remissions as did the two patients treated elsewhere. Thus, we have had the opportunity of observing 14 patients who made satisfactory responses to initial busulphan therapy (Table I) Details of these patients

TABLE I.—*Initial Response to Busulphan*

Previous therapy	Number of cases	Response	
		Good	Poor
Nil	16	14	2
Radiotherapy—Resistant	4	—	4
—Non-resistant	2	2	—
6-mercaptopurine	2	1	1
Triethylene melamine	1	1	—
Total	25	18	7

TABLE II.—*Response to Initial Therapy with Busulphan*

Case No.	Age	Sex	Initial W.C.C./ cu. mm.	Initial response to busulphan	Subsequent course		Survival from diagnosis (in months)	Comment
					W.C.C./ cu. mm.	Dose in mg. daily		
1	26	M.	112,000	Good	8–26,000	0·5–1·5	49	Terminal blastic crisis.
2	61	M.	293,000	Good	3–72,000	Nil–6	59	Episode of marrow failure. Terminal blastic crisis.
3	59	F.	338,000	Good	10–31,000	0·5–1·5	14	Skin pigmentation. Terminal blastic crisis.
4	63	F.	309,000	Poor	—	—	¼	Initial thrombocytopænia. Haemorrhage.
5	67	M.	88,000	Poor	6–30,000	—	3	Initial thrombocytopænia. Haemorrhage.
6	53	M.	164,000	Good	8–24,000	0·5–1·0	22	Myocardial infarct.
7	49	F.	270,000	Good	12–24,000	0·5–2·0	21	Carcinoma breast.
8	67	F.	264,000	Good	5–34,000	0·5–1·5	31+	Well controlled.
9	66	F.	138,000	Good	9–18,000	0·25–1·5	14+	Well controlled.
10	50	F.	295,000	Good	9–29,000	0·5–2·0	26+	Well controlled.
11	53	F.	28,000	Good	7–14,000	—	19+	Continued remission.
12	42	M.	88,000	Good	8–30,000	Nil–1·5	31+	Subsequently well controlled.
13	65	M.	215,000	Good	11–26,000	0·25–1·0	10+	Thrombocytosis.
14	34	M.	98,000	Good	4–14,000	0·25–1·0	7+	Well controlled.
15	57	F.	50,000	Good	10–16,000	0·5–2·0	6+	Thrombocytosis.
16	24	F.	409,000	Good	10–302,000	Nil–6	24+	Subsequent resistance to busulphan and radiotherapy.

are summarized in Table II. A clinical response to therapy was usually evident within two weeks and preceded haematological improvement in all cases. This was first manifested by a fall in the total white cell count, the disappearance of primitive granulocytes, and later followed by a rise in haemoglobin concentration.

The time required to achieve remission, i.e. to reduce the white cell count to under 20,000 per cu.mm., varied from 24 to 100 days and the total dosage of busulphan given in this initial period varied from 102 to 380 mg.

The only two patients in whom we were unable to produce a remission, both presented with purpura associated with severe thrombocytopænia. In one of these (case 4) therapy did not affect the white cell count, nor was the platelet count affected. In the other (case 5) therapy succeeded in lowering the white cell count, but as in the previous patient, the platelet count remained unaltered. These patients survived the initiation of therapy by two weeks and three months respectively and both died from uncontrollable gastro-intestinal haemorrhage.

Of the 14 patients making satisfactory responses nine are still alive and five dead. Six of these nine survivors were given continuous therapy and are still in remission, having been controlled for periods varying from six months to 31 months. The maintenance dose of busulphan varied from 0·5 mg. on alternate days to 2·0 mg. daily. These six patients are in good health and have been able to resume normal activities.

In the remaining three patients who still survive, busulphan was discontinued after the initial response. One of these (case 12) was initially given a 14-day course of busulphan. He first came under our observation five months later, at which time he was still in remission with a white cell count of 8,700 per cu.mm. Five months later, when the white cell count had risen to 30,000 per cu.mm. busulphan was recommenced. His response was satisfctory and over the past 20 months he has been on maintenance therapy and has remained in clinical and haematological remission. The second patient (case 16) initially treated elsewhere with good response, failed to re-attend for review for nine months. When first seen by us she was in clinical and haematological relapse with a haemoglobin of 9·2 g./100 ml. and a white cell count of 247,000 per cu.mm. Busulphan therapy was re-instituted and although the white cell count fell to 16,000 per cu.mm. there was no improvement in haemoglobin concentration or diminution of splenomegaly. Splenic irradiation was given, which produced a remission lasting only three months. Further busulphan and splenic irradiation have proved unsatisfactory in controlling the disease although the patient still remains in moderate health. The third patient (case 11) had busulphan for one month and achieved a complete remission which has lasted for 18 months, the white cell count remaining between 7–14,000 per cu.mm., without any further therapy.

Five patients are dead: two (cases 6 and 7) died from causes unrelated to their leukaemia, namely myocardial infarct and carcinoma of breast, while the remaining three died from their chronic granulocytic leukaemia.

The two former patients (cases 6 and 7) had been in satisfactory remission on continuous drug therapy for 22 and 21 months before death. A short summary of the history of each of the three patients who died from their leukaemia follows:

Case 1, a male aged 26 years, survived the institution of therapy for 49 months. Until two months before his death he was able to remain at work and lead a substantially normal life with a controlled white cell count. Terminally his leukaemic process became frankly "blastic" and he died from cerebral haemorrhage. It is of interest to note that after he had been on continuous therapy for 36 months he became the father of a normal child.

Case 2, a male aged 61 years, after initial remission was controlled on a maintenance dose of 0·5 mg. daily for 12 months, before the white cell count began to

rise again. Fluctuations in the white cell count then necessitated frequent changes in busulphan dosage over the next six months, the dose reaching 6 mg. daily for a short period of time before a steady therapeutic level was again reached. However, the white cell count then continued to fall and despite withdrawal of the drug a peripheral pancytopænia developed. Five months after cessation of therapy the haemoglobin level was 7·4 g./100 ml., leucocytes 3,500/cu.mm. and platelets 50,000/cu.mm. This episode of partial marrow failure lasted eight months during which time the haemoglobin was maintained by blood transfusions. Recovery then ensued and was followed by a remission lasting 12 months, the haemoglobin concentration, white cell count and platelets during this period being within normal limits. The leukaemic process then again became evident, and although haematological control by busulphan was unsatisfactory and frequent changes of dosage were needed, the patient remained in fairly good health. Terminally a blastic phase developed and death from haemorrhage ensued 59 months after diagnosis.

Case 3, a female aged 59 years, was well controlled for 10 months on doses varying from 0·5 to 1·5 mg. daily. From then until her death, four months later, control was unsatisfactory, the white cell count rose as did the number of blast cells in the peripheral blood, and she needed frequent transfusions. Coincidental with the worsening of her condition she was noted to be developing generalised brownish skin pigmentation which persisted, despite treatment with nicotinamide, until her death. Autopsy revealed leukaemic deposits in the adrenal glands but as she also had hypertension throughout the last few months of her life, it was felt unlikely that she suffered from secondary Addison's disease. Generalised pigmentation has been described by other authors as a side effect of busulphan therapy, but this is the only instance we encountered.

In these three patients the leukaemic process terminated in the same manner, i.e. the development of an acute leukaemic phase. Once this phase appeared, further therapy either with busulphan, 6-mercaptopurine or steroids had no effect.

Group (b)

Nine patients have been observed who, before commencing busulphan, were treated by radiotherapy (6 cases), 6-mercaptopurine (2 cases) and triethylene melamine (1 case) (Table III).

Of the six patients given initial radiotherapy, two, after making a satisfactory response, were changed electively to busulphan for maintenance therapy, whilst four were given busulphan when they had become radio-resistant. Case 19, a male aged 35 years, had two courses of splenic irradiation, each of which produced a good remission lasting 12 months. Twelve months after the second course he was again in relapse and treatment with busulphan in standard dosage was started, achieving a complete remission in two months. For the next 22 months he has been satisfactorily controlled on doses of busulphan ranging from 0·5 mg. to 1·0 mg. daily. Case 21, a female aged 24 years, made a satisfactory response to an initial course of splenic irradiation. Fifteen months later, when she was in relapse her therapy was changed to busulphan which induced a remission in four weeks. This continued for five months when she developed lobar pneumonia and died.

Four patients (cases 22, 23, 24 and 25), who had become resistant to radiotherapy, were subsequently given busulphan in standard dosage and in none of these patients was satisfactory control achieved ; in all the white cell count was

TABLE III.—*Response to Busulphan after Previous Therapy*

Case No.	Age	Sex	Previous therapy	W.C.C./ cu.mm. prior to busulphan	Response to busulphan	Survival after busulphan (in months)	Survival from diagnosis (in months)	Comment
17	18	M.	6-mercaptopurine	319,000	Good	6+	9+	Alive and well.
18	18	F.	6-mercaptopurine	84,000	Poor	18+	21+	Thrombocytosis of 3,000,000/ cu. mm.
19	35	M.	Radiotherapy	171,000	Good	24+	60+	Alive and well.
20	40	F.	Triethylene melamine	93,000	Good	10	23	Died. Blastic termination.
21	24	F.	Radiotherapy	63,000	Good	6	21	Died. Lobar pneumonia.
22	36	F.	Radiotherapy	38,000	Poor	2	108	Died. Blastic termination.
23	41	F.	Radiotherapy	63,000	Poor	11	78	Died. Cachexia.
24	59	F.	Radiotherapy	91,000	Poor	7	43	Died. Blastic termination.
25	59	F.	Radiotherapy	87,000	Poor	5	65	Died. Cachexia.

reduced but this was not associated with a rise in haemoglobin level or clinical improvement. These patients all died from their leukaemia and none survived the onset of chemotherapy by more than 11 months. In two patients, death was preceded by a blastic crisis and two died in a cachetic state.

Two patients (cases 17 and 18) given 6-mercaptopurine initially obtained only partial remissions and three months after diagnosis were started on busulphan. One (case 17) made a satisfactory response to this, and his subsequent clinical course has been uneventful for six months, during which time his white cell count has varied between 10,000 and 25,000 per cu.mm. The other patient, a female aged 18 years (case 18) did not make a satisfactory response to busulphan. On this drug her white cell count tended to fluctuate and her gross splenomegaly was not influenced. She was treated with splenic irradiation to which she made only a temporary response. She is still alive and in fair health 21 months after diagnosis, although in the past 12 months her condition has only been partially controlled by the use of both chemotherapy and radiotherapy.

The patient (case 20) initially given triethylene melamine was a female aged 40 years. She had made a moderate response to this treatment, but 13 months after diagnosis was in clinical and haematological relapse. A course of busulphan produced a satisfactory remission which was maintained for two months. She then failed to re-attend the clinic and when next seen six months later, she was in a moribund state. Her peripheral blood examination showed that the leukaemic process had undergone blastic transformation and death ensued rapidly.

The most striking feature about this group concerns those patients who had received previous radiotherapy ; those who were radio-resistant had a uniformly poor response to busulphan, whereas those who were changed before resistance developed had good and maintained remissions.

Effect of Busulphan on the Platelet Count

Platelet counts were performed on all patients at the time of diagnosis and also on each subsequent review. All platelet counts given are those obtained from venous samples using the direct counting method ; the normal range in this

laboratory is taken as 200–500,000 per cu.mm. An initial platelet count in all but three of our 25 patients lay within the range of 150,000 to 500,000 per cu.mm. ; two of these presented with thrombocytopænia and died shortly after diagnosis, whilst the third had an initial platelet count of 794,000 per cu.mm. In only six patients were significant variations in the platelet count recorded throughout the course of their illness, not associated with the development of a terminal blastic phase. One patient (case 2) developed thrombocytopænia associated with partial marrow failure and five patients developed thrombocytosis. This term as used here is defined as a platelet count of over a million on at least two consecutive occasions.

Case 13, a male aged 65 years, had initial white cell count of 215,000 per cu.mm. and a platelet count of 794,000 per cu.mm. After five to seven weeks treatment before remission was established, when the white cell counts lay between 47,000 and 51,000 per cu.mm., the platelet count lay between $1{\cdot}36 \times 10^6$ and $1{\cdot}68 \times 10^6$ per cu.mm. The daily dose of busulphan was reduced at this time and since then the platelet count has fallen. Over the period three to six months after the start of the therapy, when the white cell count has been at the desired level, the platelet count has varied from 316,000 to 646,000 per cu.mm.

Case 15, a female aged 57 years, had a white cell count of 50,500 per cu.mm. and a platelet count of 248,000 per cu.mm. when first seen some six months ago. After four weeks chemotherapy, the white cell count was 14,000 per cu.mm. and the platelet count 714,000 per cu.mm. She has been kept on busulphan continuously and the white cell count has been maintained between 10,000 and 16,000 per cu.mm. After 12 and 14 weeks therapy the platelet counts have been 1×10^6 and $1{\cdot}2 \times 10^6$ per cu.mm. respectively.

Case 17, a male aged 18 years, had a white cell count of 319,000 per cu.mm. and the platelet count was 248,000 per cu.mm. He was initially given 6-mercaptopurine which produced only a partial response and when changed to busulphan some three months later his white cell count was 100,300 per cu.mm. and platelet count was 346,000 per cu.mm. After five to six months continuous therapy, during which time the white cell count was maintained at satisfactory levels, the platelet count has risen to between $1{\cdot}23 \times 10^6$ and $1{\cdot}34 \times 10^6$ per cu.mm. respectively.

Case 18, a female aged 18 years, was initially treated with 6-mercaptopurine with partial success. She was then given busulphan and after three to four months continuous therapy, the platelet count had risen from 438,000 to between $1{\cdot}39 \times 10^6$ and $1{\cdot}75 \times 10^6$ per cu.mm. respectively. Coincidentally the white cell count was reduced, but the gross splenomegaly was not influenced and she was given splenic irradiation. This reduced the platelet count only very temporarily. Since that time she has been given busulphan, radio-active phosphorus, X-ray therapy and triethylene melamine, but none of these agents has succeeded in substantially lowering the platelet count. During the 13 months previous to review platelet counts varying from $1{\cdot}35 \times 10^6$ to 3×10^6 per cu.mm. have been recorded.

Case 16, a female aged 24 years, diagnosed elsewhere, had a satisfactory response to busulphan and initially the platelet count varied between 250,000 and 400,000 per cu.mm. When first seen by us she was in clinical and haematological relapse with a white cell count of 247,000 per cu.mm. and a platelet count of $1{\cdot}26 \times 10^6$ per cu.mm. She was given busulphan which reduced the white cell count but did not affect the high platelet count or the gross splenomegaly. Radiotherapy was

given which produced a short but good response in both the leucocyte and platelet counts. When the white cell count started to rise, the platelet count did so also and since that time (some nine months ago) busulphan and radiotherapy have not significantly altered the platelet count which remains over 1×10^6 per cu.mm.

DISCUSSION

All the currently available regimes of therapy for chronic granulocytic leukaemia are purely palliative. The points on which the different regimes may be judged are the initial remission rate, efficacy of control, incidence of side effects and effect on survival. We will now discuss our results under these headings.

In the present series of 25 patients given busulphan for chronic granulocytic leukaemia, 18 made satisfactory responses, and this incidence of initial remissions is similar to that reported by other authors (Galton, 1953; Louis, Limarzi and Best, 1956; Blackburn, King and Swan, 1956; Greig, 1956; Turesson, 1957).

Seven patients failed to make a satisfactory initial response to busulphan and of these, four had had previous radiotherapy and were resistant to it, two had thrombocytopænia at the time of diagnosis and one had been previously treated with 6-mercaptopurine to which she only partially responded. Our failure to achieve any significant remissions in the four patients who were resistant to radiotherapy was disappointing as this had not been the experience of others (Galton, 1953; Blackburn *et al.*, 1956; Louis *et al.*, 1956; Hyman and Gellhorn, 1956). It is of interest to note that two patients responding poorly to busulphan were subsequently given splenic irradiation but made only a partial and very temporary response to this therapy. In neither of the two patients with initial thrombocytopænia was this aggravated by busulphan therapy, although both died from haemorrhage. Greig (1956) treated two similar cases with busulphan and found the thrombocytopænia to be accentuated. However, Hayhoe (1960) and Haut, Abbott, Wintrobe and Cartwright (1961) have by the use of busulphan induced remissions in patients presenting with thrombocytopænia. Thus, it would appear that although the response of such patients to busulphan is variable, as radiotherapy is definitely contra-indicated, a course of this drug is worthy of trial.

Patients in whom disease is controlled by busulphan show very little clinical or haematological evidence of their leukaemic process. They are able to resume full activities, their haemoglobin level and leucocyte counts are in the normal range and the only abnormality in the differential white cell count is a slight shift to the left in the granulocytic series and perhaps a slight increase in the number of basophils present; splenomegaly may disappear or be greatly diminished.

Busulphan may be given either continuously or intermittently. Continuous therapy is given in an attempt to maintain the white cell count between 10 and 20,000 per cu.mm. and thus achieve a relatively stable haematological and clinical course. The theory in favour of intermittent therapy in which the drug is withdrawn when the white cell count falls to under 10,000 per cu.mm. to be reintroduced when relapse occurs is that this regime may delay the emergence of drug resistance.

Our experience of intermittent therapy is very limited as in only four cases has the drug been withdrawn for periods of greater than four weeks. In one case the withdrawal of therapy was necessitated by the development of peripheral pancytopænia; in this case once the erythroid and megakaryocytic elements

recovered there was a remission of the leukaemic process for a further 12 months. Such prolonged remissions following toxic marrow damage have been reported by other authors. In two patients the drug was withheld because the white cell count had fallen to under 10,000 per cu.mm.; one of these patients is still in complete haematological remission 18 months later and in the other patient the remission lasted for 11 months. In this latter case the drug was recommenced at the earliest sign of haematological relapse and subsequent control on maintenance therapy has been satisfactory. In the remaining patient in whom, because of her failure to re-attend, busulphan was not given until frank clinical relapse was established, the subsequent response was unsatisfactory. We have, however, been impressed by the ability of maintenance therapy to keep these patients in very good clinical and haematological control and as Galton, Till and Wiltshaw (1958) and Bethell (1958) have shown that continous therapy is not associated with the earlier emergence of drug resistance we would feel that this method of therapy is the one of choice.

Compared to the earlier chemotherapeutic agents the side effects of busulphan are surprisingly few. Gastro-intestinal intolerance occurs very rarely and although reported recently by Ghose and Chatterjea (1960) in six out of 46 patients treated by them we have not encountered it in any case.

The most important side effect is depression of the marrow elements. The incidence of this complication depends largely on the dose of the drug used. It was seen more frequently in the earlier days of busulphan therapy when daily doses of the order of 16 mg. were given. It is now, however, generally agreed that the only advantage of these larger doses is to induce a more rapid response and that equal benefit can be derived from the less intensive therapy as used in the present series. Hayhoe and Kok (1957) have reported a patient who unexpectedly developed marrow failure while on a moderate dosage and a similar case previously reported by one of us (Nelson and Lowry, 1957) is included in the present series. The possibility of marrow failure occurring in patients on moderate doses of the drug makes it important that all patients on busulphan should be reviewed at least once a month and at each visit their haemoglobin level, leucocyte and platelet counts should be determined.

One case of generalised skin pigmentation was encountered after busulphan had been given for ten months. This complication had been previously noted (Dameshek and Gunz, 1958; Hayhoe, 1960) but the aetiology of this condition is not clear. Cutaneous pigmentation may occur in patients with a wide variety of malignant diseases and it may well be that this is a complication more of the underlying leukaemic process rather than of the therapy.

The effect of busulphan on the sex glands of the experimental animal and the human is not yet clearly established. In the experimental animal, e.g. the rat, the drug causes infertility by interfering with spermatogenesis (Jackson, Fox and Craig, 1959) and sterility and azoospermia in the human have been recorded by Wilkinson and Turner (1959). It is of interest that one of our patients, aged 26 years, became the father of a normal child after continuous busulphan therapy for three years. Amenorrhoea has been described as a side effect of busulphan therapy by Ghose and Chatterjea (1960) who noted it in six of 13 patients and by Louis *et al.* (1956) who noted it in all four of their female patients in the relevant age group. Six of our female patients were in the reproductive span of life. In two of them amenorrhoea was present before diagnosis persisted, whilst in the

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other four no disturbance in menstruation was noted. These findings would tend to suggest that at the level of dosage employed in the present series little effect on the sex glands, either in the male or female, occurs.

The present series gives very little information as to the effect of busulphan on survival time of patients with chronic granulocytic leukaemia. We have only three patients who made an initial response to busulphan, were maintained on the drug and subsequently died from their leukaemia; these patients survived diagnosis by 14, 49 and 59 months. Amongst our patients who died from leukaemia but at some stage received therapy other than busulphan the longest survival was 108 months. Of our patients who still survive the longest interval which has elapsed since diagnosis is 60 months. Thus the survival time of none of our patients is remarkable as Minot *et al.* (1924) found that occasional untreated patients survived for up to nine years. Galton *et al.* (1958) and Ghose and Chatterjea (1960) have found that busulphan, like radiotherapy, does not significantly prolong life and our limited experience of the drug would lead us to agree with them. Wilkinson and Turner (1959) suggest that the somewhat paradoxical situation whereby busulphan which is undoubtedly effective in producing complete remissions but does not appear to significantly prolong life, may be due to the fact that patients so treated have a greater frequency of blastic crises. Blackburn *et al.* (1956) were the first to suggest that the use of busulphan might be attended by a greater frequency of this complication. In our present series eight patients who initially responded to some form of therapy, subsequently died from their leukaemia. In six of these patients the terminal event was blastic transformation of the leukaemic process and the remaining two died from anaemia and cachexia. Blastic crises were reported by Shimkin, Mettier and Bierman (1951) in 16 out of 64 untreated cases studied at autopsy, an incidence of 25 per cent, and Louis, Best and Limarzi (1957) found an incidence of 59 per cent in a series of 27 cases. Although Wilkinson and Turner (1959) came to the conclusion from their review that the incidence was increased, Haut *et al.* (1961) found, from a review of the literature and their own series, that approximately 50 per cent of patients with chronic granulocytic leukaemia, whether untreated or given busulphan, developed a blastic termination. That busulphan increases the frequency of blastic termination cannot be regarded as proven and it seems likely that this point will be most difficult to confirm owing to the lack of information on the manner of termination of untreated patients with chronic granulocytic leukaemia.

It is well recognised that in chronic granulocytic leukaemia the platelet count is very variable and in our series of 25 patients the platelet counts lay between 40 and 794,000 per cu.mm. at the time of diagnosis. The effect of busulphan on the platelet count in chronc granulocytic leukaemia is unpredictable but in the majority of patients it remains within normal limits. Many authors have emphasized the possibility of this drug having a toxic effect on the megakaryocytes (Hyman and Gellhorn, 1956; Louis *et al.*, 1956; Turesson, 1957) and indeed we have seen one such case. However, the more striking effect which we encountered in the present series has been thrombocytosis which occurred in five of our 25 patients. In one patient the thrombocytosis was first noted at the time of frank haematological relapse after an initial satisfactory remission with busulphan. In the other four patients the high platelet counts developed whilst on continuous busulphan therapy and were noted at a time when the leucocyte counts were either partially or completely controlled. In no case was the thrombocytosis associated

with any clinical manifestations. It is difficult to understand why in some cases busulphan may cause a toxic thrombocytopænia whilst in others, in which as judged by the white cell count the drug appears to be effective, thrombocytosis develops.

In our experience, busulphan induced remission in a high percentage of patients with chronic granulocytic leukaemia and in the majority of these patients this was maintained by the use of continuous therapy. These benefits were obtained with few serious side effects, although unexpected marrow failure occurred in one patient. The incidence of remissions obtained by the use of busulphan is equal to that following radiotherapy (Ghose and Chatterjea, 1960) and it is generally agreed that neither regime significantly prolongs survival. However, as drug therapy is easier to administer and less unpleasant for the patient, we consider that busulphan is the initial treatment of choice for chronic granulocytic leukaemia. The fact that neither regime prolongs survival would suggest that neither radiotherapy nor chemotherapy alters the basic defect, whatever that may be, in chronic granulocytic leukaemia and curative therapy for this condition must await further advances.

SUMMARY

The results of treatment of 25 cases of chronic granulocytic leukaemia by busulphan are recorded. Of 16 patients given this drug as initial therapy, 14 made satisfactory responses; the two who failed to respond had severe thrombocytopænia at the time of diagnosis. Six patients had previous radiotherapy; two were changed electively to busulphan for maintenance therapy and achieved good responses, whilst four who had become resistant to radiotherapy showed no worthwhile improvement. Maintenance therapy with busulphan proved very satisfactory, stable haematological and clinical courses being achieved in the large majority of cases.

Toxic effects were few, the only serious one being temporary marrow depression, which occurred unexpectedly in one patient. No increase in longevity was noted and there was a high incidence of terminal blastic crises. Five patients developed thrombocytosis and in four this occurred whilst on maintenance therapy with busulphan.

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REFERENCES

Bethell, F. H.—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 996.
Blackburn, E. K., King, G. M. and Swan, H. T.—(1956) *Brit. med. J.*, i, 835.
Dameshek, W. and Gunz, F.—(1958) 'Leukaemia'. New York (Grune and Stratton).
Galton, D. A. G.—(1953) *Lancet*, i, 208.
Idem, Till, Morwenna and Wiltshaw, Eve—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 967.
Ghose, S. and Chatterjea, J. B.—(1960) *J. Indian med. Ass.*, **34**, 381.
Greig, H. B. W.—(1956) *Acta haemat.*, **16**, 171.
Haddow A. and Timmis, G. M.—(1953) *Lancet*, i, 207.
Haut, A., Abbott, W. S., Wintrobe, M. M. and Cartwright, G. E.—(1961) *Blood*, **17**, 1.

HAYHOE, F. G. J.—(1960) ' Leukaemia '. London (J. A. Churchill).
Idem and KOK, D'A.—(1957) *Brit. med. J.*, ii, 1468.
HYMAN, G. A. AND GELLHORN, A.—(1956) *J. Amer. med. Ass.*, **161**, 844.
JACKSON, H., FOX, B. W. AND CRAIG, A. W.—(1959) *Brit. J. Pharmacol.*, **14**, 149.
LISSAUER, W.—(1865) *Berl. klin. Wschr.*, **2**, 403.
LOUIS J. LIMARZI, L. R. AND BEST, W. R.—(1956) *Arch. intern. Med.*, **97**, 299.
Idem, BEST, W. R. and LIMARZI, L. R.—(1957) *Clin. Res.*, **5**, 150.
MINOT, G. R., BUCKMAN, T. E. AND ISAACS, R.—(1924) *J. Amer. med. Ass.*, **82**, 1489.
NELSON, M. G. AND LOWRY, J.—(1957) *Irish J. med. Sci.*, **7**, 186.
PUSEY, W. A.—(1902) *J. Amer. med. Ass.*, **38**, 911.
SHIMKIN, M. B., METTIER, S. R. AND BIERMAN, H. R. (1951) *Ann. intern. med.*, **35**, 194.
TURESSON, D.—(1957) *Brit. J. Haemat.*, **3**, 220.
WILKINSON, J. F. AND TURNER, R. L.—(1959) *Progr. Haemat.*, **2**, 225.

STUDIES ON THE URINARY EXCRETION OF CERTAIN TRYPTOPHAN METABOLITES IN BILHARZIASIS AND ITS POSSIBLE RELATION TO BLADDER CANCER IN EGYPT

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THE fact that certain aromatic amines were ascribed as causative agents for the bladder cancer amongst dyestuff workers has now been established. Bladder cancer was also experimentally induced in animals by administration of 2-naphythylamine, 4-aminophenol, hydrazobenzene, aminoazotoluene and its dimethyl derivative and xenylamine (Hueper, 1938; Nelson and Woodard, 1953; Walpole, Williams and Roberts, 1954). The carcinoginic effect of these aromatic amines on the urinary bladder is mainly due to the excretion of certain metabolites of these amines in the urine. Some ortho-aminophenols which are normal products of endogenous metabolism of tryptophan and which naturally occur in small amounts in human urine are now known to induce bladder cancer (Allen, Boyland, Dukes, Hornings and Watson, 1957; Boyland and Manson, 1958).

In view of the high incidence of bladder cancer in Egypt together with the widespread urinary bilharzial infection it appeared desirable to find out whether any of the carcinogenic metabolites are associated with and might be responsible for the production of bladder cancer in this country.

The liver is known to be the main site of metabolism of such compounds. Variable degrees ranging from mild to severe liver disturbances have been shown to be accompanied with simple and complicated bilharzial affections (Abul-Fadl and Abdin, 1952; Mousa and El-Garem, 1959).

The role of certain enzymes particularly the β-glucuronidase in the mechanism of the production of cancer of the bladder has been frequently discussed (Boyland, Wallace and Williams, 1957; Boyland, Gasson and Williams, 1957). The liver also seems to be actively involved in the production of such enzymes.

For these reasons we felt it necessary to start an extensive study of the urinary excretion of certain tryptophan metabolites together with β-glucoronidase, acid and alkaline phosphatase as well as the blood serum levels of transaminases, alkaline phosphatases and other necessary liver function tests.

The present communication is a preliminary report on the results so far obtained concerning tryptophan metabolites excretion on a total of 220 cases comprising simple urinary bilharziasis (35) bilharzial hepatosplenomegalies (60); bladder cancer with active or bilharzial history (75) and normal subjects (50).

Table I shows the urinary excretion of certain tryptophan metabolites in the above mentioned cases.

The level of excretion of 3-hydroxy-anthranilic acid amongst Egyptians seems to be lower than that described elsewhere. This has been also confirmed by

TABLE I.—*The Excretion of Certain Tryptophan Metabolites in Normal Bilharzial and Cancer Bladder Egyptians*

	Number of cases	Volume of urine (ml./24 hr.)		Total N output (g./24 hr.)		Tryptophan metabolites excretion (mg./24 hr.)									
						Serotonin		Indol 3 acetic acid		3-OH anthranilic acid		Anthranilic acid		Kynurenin	
		Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average
Normals . .	50	650 to 1200	866	7·9 to 13·2	10·83	3·2 to 13	7·8	3·13 to 8·3	4·95	0·57 to 2·81	1·09	0·15 to 0·66	0·35	1·6 to 5·43	3·5
Simple bilharziasis	35	660 to 1400	940	7·56 to 9·95	8·45	16·81 to 29·27	2·14	1·99 to 6·4	3·7	0·35 to 6·49	2·01	0·15 to 0·63	0·35	2·4 to 5·35	3·6
Bilharzial hepatosplenomegaly with active urinary bilharziasis	50	450 to 1700	814	6·32 to 10·13	9·4	13·4 to 26·2	20·21	3 to 6·4	4·1	0·45 to 6·24	1·8	0·12 to 0·67	0·35	2·97 to 6·5	3·65
Cancer bladder with active urinary bilharziasis	40	650 to 2000	900	7·13 to 10·19	9·65	41·2 to 204·5	114·8	3 to 7·73	4·8	0·81 to 28·2	7·9	0·11 to 0·84	0·4	2·9 to 6·13	3·7

Professor E. Boyland at the Chester Beatty Laboratories where he kindly conducted a series of determinations of 3-hydroxyanthranilic acid on samples of urines preserved with acid and flown from Cairo to London. Nevertheless, a relative increase in this metabolite was shown to occur in both simple and complicated bilharziasis to about twice the average normal.

In bladder cancer with urinary bilharzial history the average excretion of this metabolite was increased to four times as much as the normal.

The changes in serotonin (5-hydroxy-tryptamine), are more interesting. Bilharzial infection whether simple or complicated caused a marked increase in serotonin excretion (about 3 times as much as the average normal). In cancer of the bladder the increase was even greater (about 10 fold).

Serotonin has also been found to increase in certain deficiency diseases, e.g. pellagra, and was found to diminish with nicotinic acid treatment. The carcinogenic activity of serotonin has not yet been established. Nevertheless its presence in such considerable amounts in urines from bladder cancer persons is very suggestive of an important nutritional factor in this disease. Such a nutritional factor would probably contribute towards the establishment and proliferation of bladder cancer.

The excretion of anthranilic acid, indol-3-acetic acid and kynurenine in bilharzial and bladder cancer patients, showed no significant variation from the excretion of these metabolites in normals.

TABLE II.—*Excretion of Tryptophan Metabolites in Normal Egyptians (L-Tryptophan 100 mg./kg. body weight)*

	Before administration of Tryptophan	After administration of Tryptophan (100 mg./kg. body weight)							
		1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	6th hr.	Rest of 24 hr.	Total excretion metabolites
Volume of urines (ml.)	900	130	70	100	85	200	110	380	1075
Serotonin 5 - OH - Tryptamine (mg.)	13·5	1·56	2·64	6	1·357	0·75	0·74	7·98	21·22
Indol 3-acetic acid (mg.)	6·75	1·16	1·5	2·5	1·9	1·9	1·1	3·26	13·32
Kynurenine (mg.)	1·12	0·46	0·88	1·56	1·08	1·0	0·95	1·9	7·77
3 - OH Anthranilic acid (mg.)	0·85	0·16	0·18	0·36	0·19	0·19	0·16	0·7	1·94

Table II gives the average excretion of the above metabolites after administration of L-tryptophan (100 mg/kg. body weight) to average Egyptian subjects. The results of this experiment with bilharzial and bladder cancer patients are not yet available.

This presentation of work in progress, suggests the use of oral and parenteral nicotinamide and its derivatives in a wide-scale trial for bilharzial and bladder cancer patients in Egypt. This might be helpful not only for prevention but also for treatment of this disease during its preliminary stages

Serotonin was estimated colorimetrically by the method of Macfarlane and others (1956).

UMI

Indol-3-acetic acid was assayed by the colorimetric method of Weissbach, and others (1959).

For 3-hydroxy-anthranilic acid, anthranilic acid and kynurenine determinations the technique described by Tompsett (1959) was followed.

Kynurenic and xanthurenic acids were determined by the method of Satoh and Price (1958).

SUMMARY

A preliminary report on the urinary secretion of certain tryptophan metabolites, in 220 cases, is presented. Results showed an increase of 3-hydroxy-anthranilic acid to twice the normal levels in cases of bilharzial affections, and to four times in bilharzial cancers of the bladder. Serotonin excretion was increased threefold and tenfold respectively. No significant changes in secretion of anthranilic acid, indol-3-acetic acid and kynurenine were detected.

The authors wish to take this opportunity to express their sincere thanks to Professor A. Haddow and to Professor E. Boyland of the Chester-Beatty Research Institute, London for supplying certain materials without which this study would not have been made possible.

REFERENCES

Abul-Fadl, M. A. M. and Abdin, Z. H.—(1952) *Proc. pharm. Soc., Egypt*, **34**, 89.
Allen, M. J., Boyland, E., Dukes, C. E., Horning, E. S. and Watson, J. G. (1957) *Brit. J. Cancer*, **11**, 212.
Boyland, E., Casson, J. E. and Williams, D. C. (1957) *Ibid.*, **11**, 129.
Idem and Manson, D.—(1958) *Biochem. J.*, **69** (in press).
Idem, Wallace, D. M. and Williams, D. C.—(1957) *Brit. J. Cancer*, **11**, 578.
Hueper, W. C.—(1938) *Arch. Path.*, **25**, 856.
Macfarlane, P. S., Dalgliesh, C. E., Dutton, R. W., Lennox, B., Nyhus, L. M. and Smith, A. N.—(1956) *Scottish med. J.*, **1**, 148.
Mousa, A. H. and El Garem, A.—(1959) *J. Egypt. Pharm. Ass.*, **42**, 444.
Nelson, A. A. and Woodard, G.—(1953) *J. nat. Cancer Inst.*, **13**, 1497.
Satoh, K. and Price, J. M.—(1958) *J. biol. Chem.*, **230**, 781.
Tompsett, S. L.—(1959) *Clin. Chim. Acata*, **4**, 3, 411.
Walpole, A. L., Williams, M. H. C. and Roberts, D. C.—(1954) *Brit. J. Industr. Med.*, **11**, 105.
Weissbach, H., King, W., Sjoerasmo and Uden Friend, S.—(1959) *J. biol. Chem.*, **234**, 81.

FLUORESCENCE MICROSCOPY IN EXFOLIATIVE CYTOLOGY

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EXFOLIATIVE cytology is a diagnostic procedure, which, having been generally accepted, is growing rapidly in importance as a means of early diagnosis of cancer. The Papanicolaou technique, developed originally to investigate gynaecological cancer, has been extensively applied to material from various sources and has collected a voluminous literature testifying to its usefulness, (Papanicolaou, 1954 ; Ayre, 1951 ; Schade, 1959). The diagnosis of the malignant cell however is, in the final analysis, subjective and it is experience and familiarity with a technique that is important, not the stain used. Philps (1954), using temporary wet films stained with methylene blue (Schuster, 1947) and permanent films stained with haemalum and eosin (Dudgeon and Wrigley, 1935), diagnosed 76·9 per cent carcinomas of lung by means of exfoliative cytology. Spriggs (1957) recommends May-Grundwald-Giemsa for the examination of serious fluids. Luckcock (1961), reporting from the Brompton Hospital, describes the use of Ehrlich's acid haematoxylin and eosin for the examination of tumour cells in blood, whereas, for the same purpose, Alexander and Spriggs (1960) find Romanowsky stains useful. The present study was undertaken to evaluate fluorescence microscopy for cytological diagnosis using acridine orange as described by von Bertalanffy, Masin and Masin (1958).

Fluorescence microscopy has been applied to cytology using berberine sulphate, acid fuchsin and the acridine group of stains (Friedman, 1950 ; Mellors, Glassman and Papanicolaou, 1952). Its use is based on the affinity of the basic fluorochrome dyes for the nucleic acids. Studies of the neoplastic cell have shown an increase in the total nucleic acids and ribonucleic acid (RNA). This increase is characteristic of cells showing a high protein synthesis and is seen in embryonic, regenerating, secretory and malignant cells. The RNA is found in the cytoplasm and the nucleoli of the affected cells. Increase in deoxyribonucleic acid (DNA), found in the chromatin of the nucleus, is seen in cells undergoing mitosis and polyploidy characteristic of advanced cancer. The fluorescent techniques described by Friedman and others have proved tedious and inadequate.

von Bertalanffy *et al.* (1958) have reviewed the literature on the role of nucleic acids in malignant cells, and the theoretical considerations for the use of basic fluorochrome stains and they have suggested the use of acridine orange in routine exfoliative cytology.

Acridine orange (AO) is a histochemical fluorochrome with a selective affinity for nucleic acids. At a concentration of 0·01 per cent and a pH of 6, recommended by von Bertalanffy, the DNA fluoresces yellow to whitish green, and the RNA red.

UMI

The differential fluorescence is due to varying degrees of polymerization of the nucleic acids, and makes possible the assessment of relative amounts of RNA and DNA in the cell. The increased RNA of the malignant cell is reflected in an increased brilliance, so that the differential fluorescence of the malignant and normal cells allows a comparison of the total concentration of nucleic acids in the various cells in the preparation. In addition, the morphological features of the cell are clearly visualised.

The advantages offered by this technique (hereafter named AOFM) are twofold. Firstly, the rapidity of processing and screening a specimen results in a saving of time, attractive to a rapidly growing and increasingly busy department. Secondly, it adds a certain amount of objectivity to the process of arriving at a diagnosis. These considerations led to an attempt to assess the value of this method in routine cytological diagnosis.

MATERIAL AND METHODS

The method used is that described by von Bertalanffy *et al.* (1958). Dart and Turner (1959) and Umiker, Pickle and Waite (1959) have described various modifications which were found to be unnecessary.

The first two months were devoted to learning what amounted to a new language. A variety of specimens were stained and examined with AOFM. Different types of cells—normal, atypical and malignant were studied. They were subsequently decolourised in two changed of 50 per cent alcohol, restained with Papanicolaou stains and the results compared with those obtained with acridine orange. The specimens thus studied are not included in the present series. Thereafter, excepting gynaecological smears, all specimens coming to the laboratory, 743 in number, were examined by both techniques. Six smears were prepared from each specimen, three were screened by the Papanicolaou technique and three by fluorescence microscopy. The person screening the AO preparations did so without having looked at the corresponding Papanicolaou smears and the results were recorded independently as positive, suspicious or negative. After the first three months, one of the three acridine orange smears was air-dried before processing and then fixed and stained in the usual way.

Apparatus

The Cook, Troughton, Simms mercury vapour lamp was used as a source of light. A blue exciter filter was fitted to the front of the lamp with a glass cell containing 5 per cent copper sulphate solution behind the filter. This could be replenished as and when necessary. Orange barrier filters were incorporated in both eye pieces.

A Cook, Troughton, Simms binocular microscope was used. Smears were screened using $\times 8$ oculars and $\times 5$ objective. In some cases of excessively cellular gastric washings and thick sputum smears, $\times 10$ objective had to be used for screening. Morphological detail was studied with a $\times 40$ objective.

The microphotographs were taken with a Leitz camera using super Anscochrome film. Trial and error showed that a 10–15 seconds exposure was adequate for a low power photograph and 20–30 seconds for a high power one.

Cellular appearances

The cellular appearances were similar to those described by von Bertalanffy *et al.* (1958) and Umiker and Pickle (1960) and are as follows (Table I). Mucus fluoresced green, bacteria orange and nucleoli when present were orange. The haemoglobin in red blood cells quenches fluorescence and they were not seen. The appearances of the cells in the air-dried preparations were identical with those that were freshly fixed. The description given above applies to a large majority of cases, but certain exceptions were encountered.

TABLE I.—*Appearances of Cells with AOFM*

	Cytoplasm	Nucleus
Benign Cells		
Leucocytes—		
Polymorph neutrophils	Brown—barely visible	Green—occasionally whitish green
Lymphocytes	Red to orange	Yellow
Superficial squamous cells	Dull olive green	Green to white
Cells undergoing squamous metaplasia	Green — occasionally very dull reddish brown	Greenish white to yellow.
Histiocytes	Brown to brownish red—inclusions may be present	Green to yellow
Mesothelial cells	Red—generally brick—occasionally orange red	Dull yellow
Columnar cells	Reddish brown or brick red	Yellow-green.
Malignant Cells		
	Cytoplasm	Nucleus
Keratinized squamous cells	Dull olive green	Bright yellow.
Poorly differentiated squamous cells	Reddish orange to flame	Bright yellow
Adeno-carcinoma	Orange to flame	Bright yellow or whitish green
Anaplastic carcinoma	Flame	Bright yellow or whitish green

Necrotic malignant cells always had a dull brownish red cytoplasm, and the nucleus varied from bright yellow to dull yellowy green irrespective of the nature of the cell. The few cases, however, in which such cells were encountered came from cases of carcinomatosis and the morphological features made the diagnosis obvious (Fig. 1).

The cytoplasm of the keratinizing squamous cell was dull green or brown and liable to be overlooked. The nucleus, on the other hand, was extremely brilliant and arrested attention. The disparity between the fluorescence of the cytoplasm and nucleus of the keratinized cell had to be kept in mind if these were not to be missed (Fig. 2). A high power examination revealed the bizarre shapes commonly seen in well differentiated squamous cell carcinoma. In the midst of a large number of pus cells and histiocytes the bright nucleus would probably not be enough to focus attention on the cell.

In thick smears of sputum with large collections of histiocytes superimposed on each other the cells appeared excessively bright and the nuclei yellow. In such cases the diagnosis was based on an examination of the single cells at the edge of the group, with the appearances and staining features characteristic of histiocytes. In addition, the thick cluster generally showed carbon inclusions and was

rejected for the purpose of diagnosis. Food particles fluoresced orange and yellow, but did not present a problem as they lack morphological characteristics of cells.

RESULTS

The 743 specimens came from 395 cases which are summarised in Table II. The miscellaneous group includes CSF, breast secretions, bronchial washings and urines.

TABLE II.—*Results of AOFM and Papanicolaou Screening*

	Serous fluids		Sputa		Gastric washings		Miscellaneous	
	AOFM	Papanicolaou	AOFM	Papanicolaou	AOFM	Papanicolaou	AOFM	Papanicolaou
Total number of cases	62	62	221	221	87	87	25	25
True positives	36	31	20	21	8	7	8	9
True suspicious	2	—	4	1	2	2	1	0
False positive	2	1	1	1	0	0	0	0
False suspicious	0	0	6	2	0	0	0	0
False negative	1	8	5	7	5	6	0	0
Percentage cases of carcinoma correctly diagnosed	97	79	82	76	67	60	100	100

The two cases (3 per cent) of serous fluids falsely diagnosed as positive were of congestive cardiac failure. The one false negative was found at autopsy to come from a case of lymphosarcoma. The cells in the effusion had in fact been diagnosed as being of the lymphocytic series, but it was not possible to say if they were malignant. Mellors (1957) has shown that DNA is not increased in human lymphoma and this could have been one reason why the cells with their narrow rim of cytoplasm were not recognized as being malignant, or at any rate, primitive.

Seven sputa were falsely diagnosed as being positive. Though this was only 4 per cent of the total number of cases examined, it represents 23 per cent of the total number of positives reported. These specimens were all from cases of bronchiectasis. It may be assumed that the exfoliated cells seen were hyperplastic and therefore fluoresced brightly. The morphological criteria of cells from cases of bronchiectasis are notoriously difficult to analyse and require greater experience than was available to us in the present survey.

EXPLANATION OF PLATE

FIG. 1.—Ascitic fluid. Necrotic malignant cells showing dull brown red cytoplasm. Case of carcinomatosis. ×700.

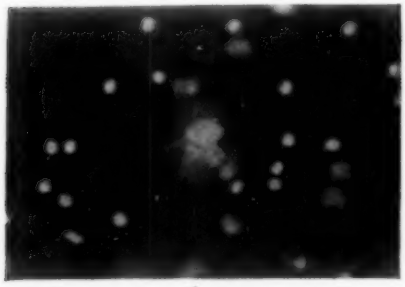
FIG. 2.—Sputum. Malignant keratinizing squamous cells with dull green cytoplasm and bright yellow nuclei. Two undifferentiated cells have orange cytoplasm. ×700.

FIG. 3.—Sputum. Malignant cells showing differential staining and fluorescence. ×250.

FIG. 4.—Ascitic fluid. Showing differential staining and fluorescence of (*a*) malignant cells: bright orange cytoplasm with large yellow nucleus; (*b*) mesothelial cells adjoining the malignant cells: dull brownigh red cytoplasm with yellow nucleus; (*c*) two histiocytes in the centre of field: brown cytoplasm with green nuclei; (*d*) polymorphs: lobulated green nucleus surrounded by very dull brown cytoplasm; (*e*) lymphocytes: bright yellow dense nucleus with a rim of red cytoplasm. ×700.

FIG. 5.—Gastric lavage. Showing morphological features of malignant cells. Note the increase in nuclear size and the coarse clumping of chromatin and nucleoli. ×700.

FIG. 6.—Ascitic fluid. Showing a malignant cluster. Note the brilliance which facilitates screening. ×250.



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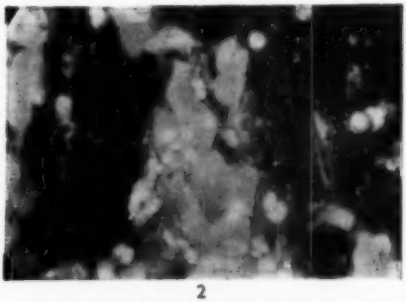

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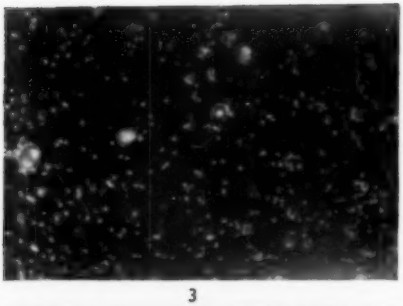

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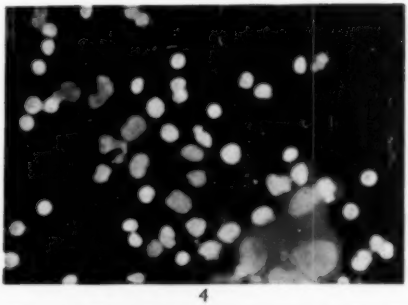

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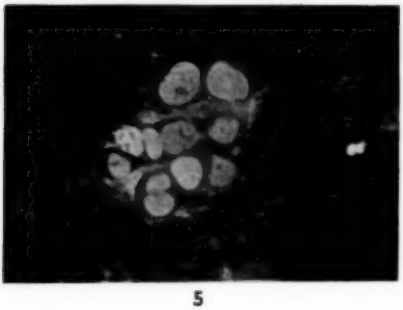

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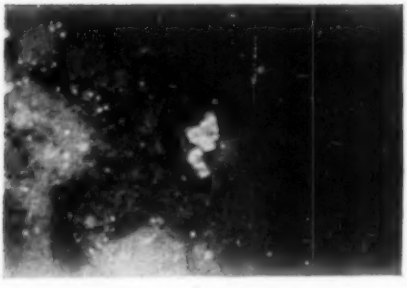

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The results obtained with fluorescence microscopy were compared with the parallel series of Papanicolaou smears (Table III). The one serous fluid correctly diagnosed by the Papanicolaou method and negative with AOFM is the case of lymphosarcoma referred to above.

TABLE III.—*Comparison of AOFM with Papanicolaou*

	Cases of carcinoma correctly diagnosed	
	FM+ Pap−	FM− Pap+
Serous fluids	8	1
Sputa	4	2
Gastric washings	1	—
Miscellaneous	0	0

Two other cases of positive serous effusion, not included in Table III, were correctly diagnosed by AOFM on cytological examination, whereas the positive " Papanicolaou " report was based on haemaloxylin and eosin sections of clots in the specimens. The two cases of sputa falsely diagnosed as negative were of keratinizing squamous cell carcinoma of the lung.

DISCUSSION

The present investigation was undertaken in search of a technique which combined rapidity with reliability. von Bertalanffy *et al.* (1958) and Dart and Turner (1959) found that AOFM compares favourably with the Papanicolaou technique, whereas Umiker and Pickle (1960), investigating carcinomas of lung, found it to be less sensitive.

All workers are agreed that the technique is more rapid than the older methods and the present investigation confirmed these findings. The staining time was 8 minutes as against the 20 minutes required by the modification of the Papanicolaou stain used in this laboratory (Crabbe, 1952). The average screening time is under five minutes. Moreover, the dark background of the AO preparation is less tiring to the eyes and, in addition to the screening of more specimens in a given time, the technician can spend longer hours at the microscope without undue fatigue.

A more important finding was the greater sensitivity of the AOFM technique in the examination of serous fluids. This was due to the clear differentiation between mesothelial cells, histiocytes and malignant cells. In serous fluids all these cells tend to be round. Vacuolation may be present within the cytoplasm of all three types. A tendency to form morulae which simulate malignant clusters is characteristic of mesothelial cells. Mitosis may be present in both mesothelial and malignant cells. Irregular nuclei are seen in histiocytes as well as malignant cells. It is these features, which the cells have in common, that make the identification of cancer cells in serous fluids difficult by the other methods. With AOFM the histiocytes have a brown cytoplasm, the mesothelial cells red and the malignant cells orange red or flame. The extreme brilliance of malignant cells is most striking in serous fluids and in the majority of cases reported above the correct diagnosis was made practically at the first glance. That acridine orange is not a " cancer " stain has been shown by Kornfield and Werder (1960), but its use is based on a

logical exploitation of a well established feature of the malignant cell and appears to be justified by the findings reported here. A factor which undoubtedly contributed to the success of the technique was the high concentration of cells which is obtained in air-dried smears. Smears which are to be stained by the Papanicolaou technique have to be freshly fixed, as these stains are not satisfactory for dried smears. Despite the use of albumin or frosted glass slides, a certain number of cells often wash off from a " Papanicolaou " smear, thus reducing the chances of making a correct diagnosis.

Last, but not least, the breath-taking beauty of the preparations, relieves the monotony of routine work.

SUMMARY

Acridine orange fluorescence microscopy was used to study 743 cytological specimens and the results were compared with the Papanicolaou method.

It was found to be more rapid, compared favourably with the Papanicolaou technique in the examination of sputa and gastric washings and was found to be more sensitive in the identification of cancer cells in serous fluids.

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REFERENCES

ALEXANDER, R. F. AND SPRIGGS, A. I.—(1960) *J. clin. Path.*, **13**, 414.
AYRE, J. E.—(1951) ' Cancer Cytology of the Uterus.' New York (Grune and Stratton).
VON BERTALANFFY, L., MASIN, M. AND MASIN, F.—(1958) *Cancer*, **11**, 873.
CRABBE, J. G. S.—(1952) *Brit. med. J.*, ii, 1072.
DART, H. L. AND TURNER, T. R.—(1959) *Lab. Invest.*, **8**, 1513.
DUDGEON, L. S. AND WRIGLEY, C. H. J.—(1935) *J. Laryng.*, **50**, 752.
FRIEDMAN, H. P. JR.—(1950) *Amer. J. Obstet. Gynec.*, **59**, 852.
KORNFIELD, H. J. AND WERDER, A. A.—(1960) *Cancer*, **13**, 458.
LUCKCOCK, E. D.—(1961) *J. med. Lab. Tech.*, **18**, 32.
MELLORS, R. C.—(1957) " Biology, Biochemistry and Pathology." In ' Analytical Pathology ', Ed., R. C. Mellors. New York (Blackiston).
Idem, GLASSMAN, R. AND PAPANICOLAOU, G. N.—(1952) *Cancer*, **5**, 458.
PAPANICOLAOU, G. N.—(1954) ' Atlas of Exfoliative Cytology.' Massachusetts (Harvard University Press).
PHILPS, F. R.—(1954) *Brit. J. Cancer*, **8**, 67.
SCHADE, R. O. K.—(1959) *Acta Cytologica*, **3**, 7.
SCHUSTER, N. H.—(1947) In Dykes ' Recent Advances in Clinical Pathology.' London (J. & A. Churchill Ltd.), p. 136.
SPRIGGS, A. I.—(1957) ' The Cytology of Effusions.' London (Heinemann).
UMIKER, W. AND PICKLE, L.—(1960) *Lab. Invest.*, **9**, 613.
Iidem AND WAITE, B.—(1959) *Brit. J. Cancer*, **13**, 398.

INCREASING THE RADIOSENSITIVITY OF TUMOURS BY COMBINED ACTION OF "DICETOL" AND RADIATION

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THE biological effects of radiation are known to be mediated in two ways: (1) effects brought about by direct "hits" on some important cell constituents, and (2) those produced by indirect action mediated through free radicals formed as a result of action on water molecules. In practice, although direct effects are seen to some extent, it is the indirect effects that predominate. It is now well established that the primary effects of radiation are as shown in the reaction sequence given below and result in the formation of H and OH radicals.

$$H_2O \xrightarrow[\text{electrons}]{\text{Knocking off}} H_2O^+ + e^- \quad . \quad . \quad . \quad . \quad (1)$$

$$H_2O^+ \xrightarrow{\text{Ionization}} H^+ + OH \quad . \quad . \quad . \quad . \quad (2)$$

$$H_2O + e^- \xrightarrow[\text{electrons}]{\text{Capture of}} H_2O^- \quad . \quad . \quad . \quad . \quad (3)$$

$$H_2O^- \xrightarrow{\text{Ionization}} H + OH^- \quad . \quad . \quad . \quad . \quad (4)$$

H and OH radicals are formed in the vicinity of tracks of ionizing radiations. Since H and OH radicals are highly reactive and have affinity for each other, their close proximity usually results in their recombination to form water as shown in the equation 5.

$$H + OH \longrightarrow H_2O \quad . \quad . \quad . \quad . \quad (5)$$

In presence of oxygen however, hydrogen radicals combine with oxygen to form perhydroxy radicals.

$$H + O_2 \longrightarrow HO_2 \quad . \quad . \quad . \quad . \quad (6)$$

Since, hydrogen radicals are removed, the danger of recombination disappears and oxidising radicals like OH and HO_2 can exist in irradiated medium for some time. There is sufficient evidence to suggest that most of the radiobiological effects arise at least in part from the effects of these free radicals. While the effect of oxygen appears to be almost universal in radiobiology, Howard-Flanders (1958) has quoted instances in which no effect of oxygen was found.

Radiosensitivity of tissues therefore depends to some extent on oxygen tension inside the cell. It also depends on the state of mitotic activity of the tissue.

Actively dividing cells appear to be more sensitive to radiation damage than the resting cells. This does not necessarily mean that resting cells are insensitive to radiation damage. Cells which have been irradiated in the resting stage and then caused to proliferate often show a great deal of damage at their first division. Malignant cells are usually rapidly dividing and hence radiation damage manifests itself much more readily. It is this property which has been exploited in radiotherapy of malignant diseases. In routine radiotherapy scrupulous care has to be taken to reduce the normal tissue-dose and at the same time increase the tumour-dose. It is with this object in view that rotation beam radiation therapy and other radiation devices have come in vogue.

Deschner and Gray (1959) have shown that the radiosensitivity increases with increase in partial pressure of oxygen up to 40 mm. of Hg or so. Beyond this the

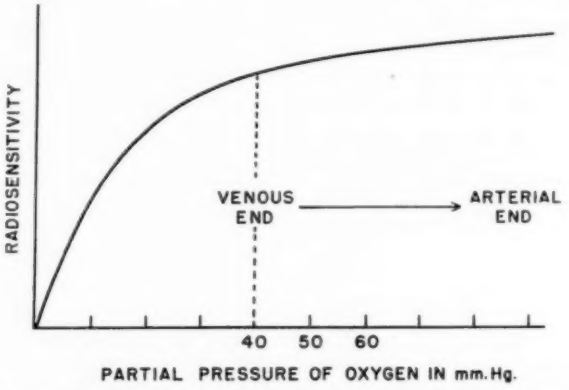


FIG. 1.—Relationship between radiosensitivity and oxygen tension.

radiosensitivity does not increase with increase in partial pressure of oxygen. This relationship of radiosensitivity of cells to partial pressure of oxygen is shown in Fig. 1. Since most of the cells, even those situated at the venous end, have a partial pressure of 40 mm. of Hg or more, their radiosensitivity is maximum.

Malignancy is characterised by rapid and unrestrained growth of neoplastic cells. As such the growth of malignant cells far exceeds that seen in the stromal cells and blood vessels. Vascularity and the supporting stroma therefore cannot cope-up with the rapid and explosive cellular multiplication of the neoplastic cells, with the result, one finds a tumour mass full of neoplastic cells without adequate blood vessels flowing through it. The entire tumour mass has thus to obtain its nutrients and oxygen from the periphery. This is schematically shown in Fig. 2. The peripheral cells are usually the first to come in contact with the flow of nutrients and oxygen from the surrounding spaces. Since these are rapidly multiplying, they " plunder " most of the nutrients and oxygen for their own proliferation and allow very little to trickle inside, with the result that a sort of a diminishing concentration gradient is set up from the periphery to the centre of the tumour mass. As the tumour gets bigger and bigger the peripherally situated cells utilize the entire supply of nutrients and oxygen

and a region of anoxia and starvation is produced in the central area of the tumour. This is probably the reason why necrosis is invariably seen in the centre of the tumour mass. (The oxygen gradient is shown in the lower half of the Fig. 2.) If such a tumour is irradiated, only the peripheral cells which have an oxygen tension round about 40 mm. of Hg will be radiosensitive. The cells lying inside, away from the periphery, will have comparatively less oxygen and hence their radiosensitivity also will be diminished. If such a tumour is irradiated, only the peripheral cells will be damaged and the cells lying inside the tumour mass will be damaged partially or not at all. This would mean that although the tumour is exposed to adequate radiation dose, it may not be possible to eradicate the entire tumour mass completely. On discontinuation of radiation treatment,

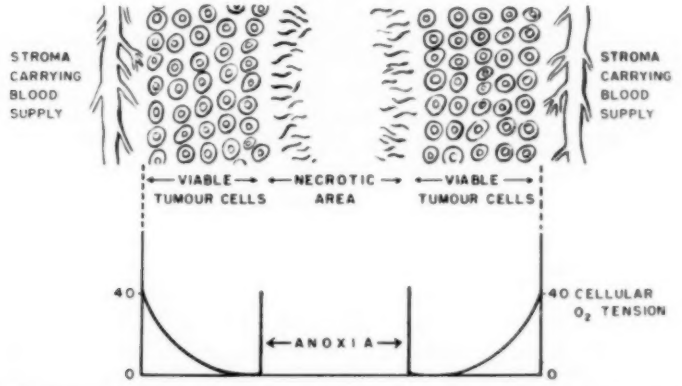


FIG. 2.—Diagrammatic sketch showing the oxygen and nutrient gradient in tumour mass. The lower half of the figure shows the oxygen gradient from the periphery to the centre (for details see text).

the cells which are lying inside, away from the periphery, would burst into mitotic activity, and the malignant growth will recur. This is probably one of the reasons why disappointing results are obtained in radiotherapy of some tumours. One way of getting over this difficulty is to increase the oxygen tension of cells lying inside and thus make them radiosensitive.

Gray *et al.* (1953), Churchill-Davidson, Sanger and Thomlinson (1957) and Sanger (1959) tried this by putting the animal or the patient in 3–4 atmospheres of oxygen. Since a gradient of oxygen is believed to exist from the periphery to the centre of the tumour mass, it was anticipated that by boosting up the oxygen pressure in the surrounding tissue it may be possible to proportionately increase the intracellular oxygen tension in the cells of the tumour mass. By adopting this procedure, these workers obtained interesting results. But the process of putting the patient in a closed chamber each time radiation is to be delivered, and increasing oxygen pressure to 3–4 atmospheres, irradiating the tumour while the patient is under the pressure and slowly releasing the pressure, required 3–4 hours and was tedius. It would be a great advantage if alternative procedure could be devised to increase the oxygen tension inside the tumour mass. This has

been attempted in our laboratory and forms the subject matter for the present communication.

The uptake of oxygen and its utilization for metabolic process mainly takes place through the Kreb's tricarboxylic acid (TCA) cycle. It also takes place to some extent through the hexose monophosphate (HMP) oxidative pathway of glycolysis. In malignant tissue it is well known that the respiration and TCA cycle are impaired and the HMP pathway is accelerated. As such the tumour cells would be using their oxygen mostly through the HMP pathway and to a lesser extent through the TCA cycle. Any substance which interferes with the HMP pathway will therefore interfere with the uptake of oxygen by tumour cells.

As a result of systematic studies, about ten compounds were synthesized in this laboratory as antimetabolites of 6-phosphogluconic acid (Sahasrabudhe, 1958). These have been tested for their anti-cancer property. Two of these—TDA (Thiophene 2 : 5 dicarboxylic acid) (Sahasrabudhe *et al.*, 1960) and " dicetol " (2 : 5 dicarbethoxy 3 : 4 dihydroxy thiophene) (Sahasrabudhe *et al.*, 1959) showed potent anti-cancer properties. They were also shown to inhibit the HMP pathway (Narurkar *et al.*, 1958). Experiments were therefore carried out to study the influence of " dicetol " on uptake of oxygen by normal and malignant cells.

MATERIAL AND METHODS

Male Wistar rats, between $2\frac{1}{2}$–3 months of age, weighing 150–200 g. were used in the present investigations for respiratory studies in normal tissues. Transplantable fibrosarcoma in Swiss mice and mammary adenocarcinoma in C_3H (Jax) and carcinosarcoma in Dba (—MTI) mice were used. Tumours were transplanted subcutaneously and allowed to grow for 13–17 days. The animals were then killed and the tumours excised and taken for respiration studies. Liver, kidney, spleen and brain from normal rats were also taken for respiratory studies. In all these cases effect of " dicetol " on glucose oxidation was studied.

Respiration studies were carried out by standard methods of Umbreit, Burris and Stauffer (1951) in Warburg apparatus. The medium in its final concentration consisted of $MgSO_4$ 3×10^{-3} M ; K-fumarate 7×10^{-5} M ; Cytochrome *c* 4 $\times 10^{-5}$ M ; KCl $1{\cdot}4 \times 10^{-1}$ M ; DPN 2×10^{-3} M ; phosphate buffer 0·02 M (pH 7·4) ; glucose 0·02 M. " Dicetol " 1 mg. in 0·5 c.c. of distilled water. Ten per cent tissue homogenate was prepared in 0·133 M KCl and 0·02 M phosphate buffer pH 7·4 in an all-glass homogenizer. 0·5 c.c. of tissue homogenate representing 50 mg. of the wet weight of the tissue was added to the chilled Warburg flask containing the above mentioned medium, making the total volume to 3·2 c.c. In the central well 0·2 c.c. of 20 per cent KOH was added for carbon dioxide absorption. The incubations were carried out for one hour at 37° C. with air as the gas phase. All calculations are done in micromoles per 100 mg. wet weight of the tissue per hour.

Combined action of " dicetol " and radiation was studied on transplantable fibrosarcoma in Swiss mice. This tumour was originally obtained by Waravdekar and Ranadive (1957) from Swiss mice treated with 6:12 dimethylbenzo (1:2b, 4:5b)-dithionaphthene. The tumour has since been maintained in Swiss mice through several serial transplantations. A large number of mice were transplanted with 0·5 ml. of homogeneous tumour suspension under asceptic conditions. After about eight days, mice having same tumour size and dimensions (visual observation) were divided into four groups. (I) The control group without any treatment

whatsoever. (II) " Dicetol " treated group received " dicetol " for a period of five days at a dose of 1 mg. twice a day (total 9 mg.) given subcutaneously. (III) Radiated group. Animals in this group received one irradiation of 42 r X-ray on the tumour. (IV) Animals in the fourth group received combined treatment of " dicetol " and radiation. Radiation was given on the 5th day after the last " dicetol " treatment. (V) In the fifth group radiation was given first on the 12th day followed by " dicetol " treatment for 5 days. Source of radiation was 250 kV X-ray machine with a filter of $\frac{1}{2}$ mm. Cu. 1 mm. Al, yielding a half value layer of $\frac{1}{2}$ mm. Cu. Radiation rate was 42 r per minute. The animals were placed in a small cardboard box, so as to restrict their movements. The rest of the animal body was shielded by covering it with lead-strips of 2 mm. thickness. Rate of tumour growth was recorded. In one experiment the animals were observed till their natural death. Whereas in another experiment the animals were killed on the 25th day after tumour transplantation, and tumour weights recorded.

RESULTS AND DISCUSSION

Effect of " dicetol " on glucose oxidation is presented in Table I. It will be seen from this table that " dicetol " interferes with the oxygen uptake of fibro-

TABLE I.—*Oxygen Uptake in μMoles per 100 mg. of Tissue With or Without " Dicetol "*

Tissue	Glucose	Glucose + " Dicetol "	Percentage of depression
Fibrosarcoma (Swiss)	5·1	1·9	64
Adenocarcinoma C_3H (Jax)	4·1	1·7	60
Carcinosarcoma Dba (—MIT)	6·9	3·0	67
Spontaneous C_3H (Jax)	4·4	2·0	55
Liver	16·0	5·2	68
Kidney	14·2	9·2	36
Spleen	2·2	1·0	55
Brain	2·0	2·0	0

Incubation time 60 minutes. Composition of medium: $MgSO_4$ 3×10^{-3} M, Pot. fumarate 7×10^{-3} M, Cytochrome c 4×10^{-5} M, KCl $1{\cdot}4 \times 10^{-1}$ M, DPN 2×10^{-3} M. Glucose 0·02 M. Phosphate buffer 0·02 M. pH 7·4. Tissue homogenate 50 mg. and " dicetol " 1 mg. per flask. Average of six experiments in duplicate flasks.

sarcoma and adenocarcinoma. In case of fibrosarcoma the oxygen uptake is lowered by 64 per cent in presence of " dicetol ". In transplantable adenomas from C_3H (Jax) animals and the carcinosarcoma from Dba (—MTI) the depression in oxygen consumption is 60 and 67 per cent respectively. In case of spontaneous adenocarcinoma the lowering in oxygen uptake was 55 per cent. Similar trend was seen in normal tissue like liver, kidney and spleen also, but not in brain. In case of liver the substrate oxidation was depressed by 68 per cent in presence of " dicetol ".

It is obvious that " dicetol " interferes with the uptake of oxygen by neoplastic and normal cells. This suggested that " dicetol " in addition to being an antimetabolite of HMP pathway also possessed the property of interfering with the respiratory chain.

The effect of combined action of " dicetol " and radiation is shown in Fig. 3. It will be seen that when " dicetol " is given first followed by radiation the tumour

growth is markedly inhibited. Neither " dicetol " nor radiation alone was effective in inhibiting the tumour growth. In Fig. 3 one finds that most of the control group animals die by 26th–28th day after tumour transplantation. Those receiving either " dicetol " treatment or the radiation exposure die by the 32nd and 34th day (average) respectively. While the animals receiving " dicetol " followed by radiation exposure live for much longer periods. Both radiation and " dicetol "

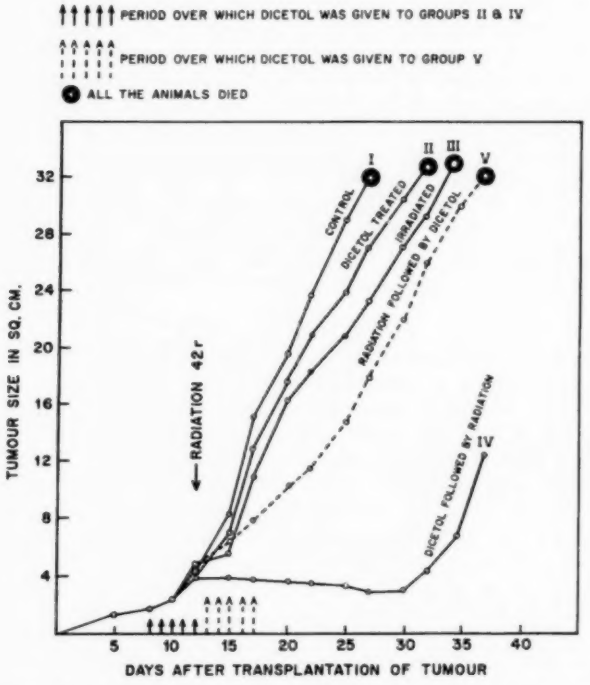


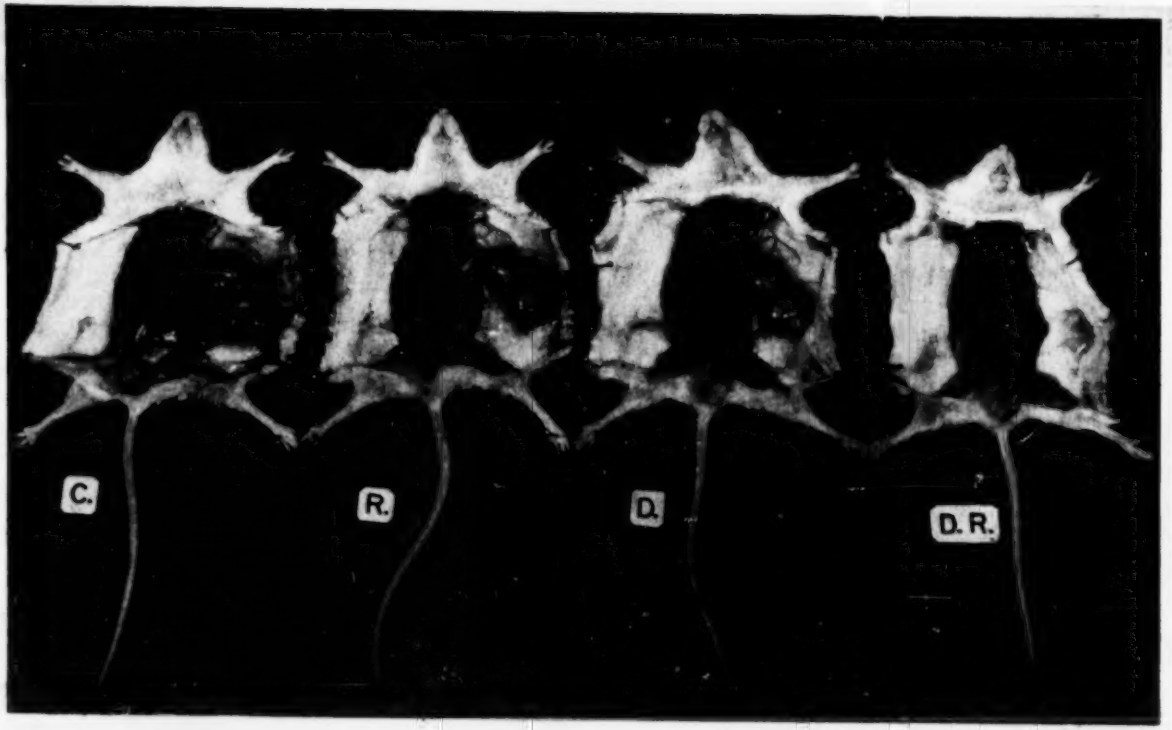
FIG. 3.—Combined action of " dicetol " and radiation on tumour growth ; fibrosarcoma in Swiss mice.

Note inhibition of tumour growth in animals receiving " dicetol " first followed by radiation (Group IV). Giving radiation first followed by " dicetol " does not seem to inhibit tumour growth (Group V). This suggests that the effect is not merely additive.

The tumour sizes have been evaluated by measuring the contours along the length and breadth of the tumour mass. Hence the figures along Y axis do not represent the absolute areas of tumour masses, but give an indication of the relative sizes.

EXPLANATION OF PLATE.

FIG. 4.—Representative mouse from each of the four groups described in the text. C : mouse from the control group without any treatment. R : mouse from Group III which was given one dose of radiation 42 r on the tumour on 12th day after transplantation. D : mouse from Group II. " Dicetol " was given for five days. Daily dose 2 mg. per mouse. D.R. : mouse from Group IV which received " dicetol " for five days followed by one exposure of radiation on the tumour on the 12th day. All animals were killed on the 25th day after transplantation. Note the marked inhibition in the group receiving " dicetol " followed by radiation (D.R.).



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are anti-cancer substances and so the result seen in group IV may be ascribed to additive effects. In group V where, " dicetol " was administered after radiation exposure, no inhibition of tumour growth was seen, suggesting that the effect of " dicetol " followed by radiation is not merely additive.

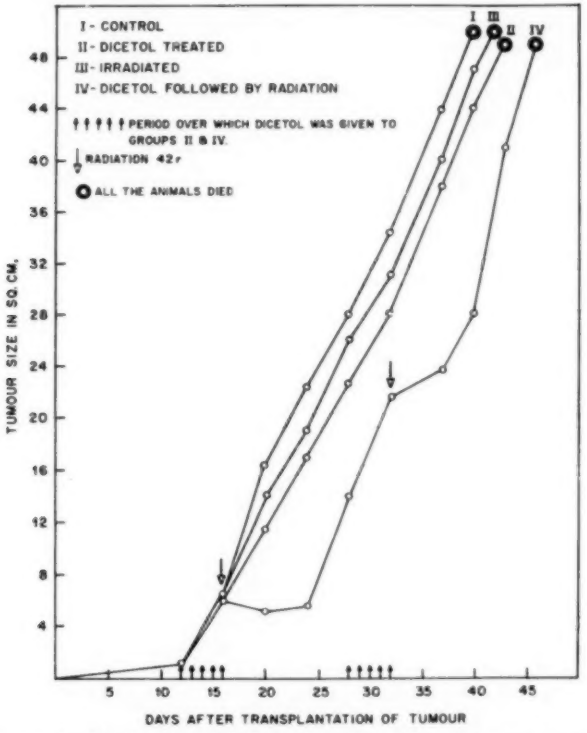
I - CONTROL
II - DICETOL TREATED
III - IRRADIATED
IV - DICETOL FOLLOWED BY RADIATION
PERIOD OVER WHICH DICETOL WAS GIVEN TO GROUPS II & IV.
RADIATION 42r
ALL THE ANIMALS DIED
I III II IV
TUMOUR SIZE IN SQ.CM.
DAYS AFTER TRANSPLANTATION OF TUMOUR

FIG. 5.—Combined action of " dicetol " and radiation on tumour growth : adenocarcinoma in $C_3H_{(Jax)}$ mice.
Note inhibition of tumour growth in animals receiving " dicetol " first followed by radiation (Group IV).

The average tumour weight of the animals, which were treated as before but killed on the 25th day of transplantation, are shown in Table II. The average

TABLE II.—*Influence of Various Treatments on Average Tumour Weights (Fibrosarcoma)*

Group	Treatment	Weight (g.)
I	Control	5·65
II	" Dicetol " (for 5 days)	4·83
III	Radiation once	4·06
IV	" Dicetol " for 5 days followed by radiation once	1·23

Average of at least six animals in each group. Animals killed 25 days after transplantation.

tumour weight (1·23 g.) in group IV was markedly lower than the other groups. As against this the tumour weights in the control, " dicetol " treated and radiated groups were 5·65 g., 4·83 g. and 4·06 g. respectively (Fig. 4).

It will be seen from Fig. 3 that the tumour growth was arrested for 18 days after a single exposure of the tumour mass with a radiation dose as low as 42 r. While it is probable that the transplanted fibrosarcoma used in the present investigation in Swiss mice was very radiosensitive, it may also be possible that the part of the arrest of tumour growth may be due to an immunity reaction between the host and the tumour transplant. The fact that this is so is substantiated with the results presented in Fig. 5. Fig. 5 shows the results of experiments in which C_3H(Jax) breast tumours were transplanted into inbred strain of C_3H(Jax) mice. There was a complete inhibition of tumour growth in this group which lasted for eight days (18 days in Swiss mice). The inhibition of tumour growth restarts after 30th day in Swiss mice, and 24th day in the C_3H(Jax) mice respectively. With a second exposure in C_3H mice the survival period was not as significantly extended as was seen in Swiss mice, although slowing down of tumour growth was definitely seen.

A method to render all the cancer cells in a tumour mass radiosensitive has been wanting for a long time. The results of the present investigations seem to demostrate that pretreatment with " dicetol " may prove beneficial in this respect. Combined treatment of " dicetol " followed by radiation has been shown to have far better results than " dicetol " or radiation given alone. Further, " dicetol " has been shown to be non-toxic in rats, mice, and also in humans. As such pretreatment with " dicetol " may help in extending and augmenting the usefulness of radiation therapy in the control of malignant diseases.

SUMMARY

A method to render radiosensitive all the cancer cells in the tumour mass has been presented. Radiation sensitivity of tumours was shown to be augmented by pretreatment with " dicetol ". Since " dicetol " is non-toxic in therapeutic doses it is suggested that combined treatment with " dicetol " and radiation may help in increasing the therapeutic effectiveness of radiation therapy in the treatment of malignant diseases.

The work presented in this communication originated in the discussions with Dr. L. H. Gray, F.R.S., Director of the British Empire Cancer Campaign Unit of Radiobiology, Mount Vernon Hospital, Northwood, Middlesex, while he was on a short visit to Bombay. The authors take the opportunity of thanking Dr. Gray for his valuable suggestions. One of us (K.N.G.) thanks the Dorabjee Tata Trust for the award of a Research Fellowship.

REFERENCES

Churchill-Davidson, I., Sanger, C. and Thomlinson, R. H.—(1957) *Brit. J. Radiol.*, **30**, 406.

Deschner, E. E. and Gray, L. H.—(1959) *Radiation Res.*, **11**, 115.

Gray, L. H., Conger, A. D., Ebert, M., Hornsey, S. and Scott, O. C. A.—(1953) *Brit. J. Radiol.*, **26**, 638.

HOWARD-FLANDERS, P.—(1958) 'Physical and chemical mechanisms in the injury of cells by ionizing radiation.' Advances in Biology and Medical Physics. Ed. C. A. Tobias and L. H. Lawrence. New York (Academic Press) 6 533.

NARURKAR, M. V., NERURKER, M. K., SAHASRABUDHE, M. B. AND TILAK, B. D.—(1958) Proceedings of the Second United Nations International Congress on the Peaceful Uses of Atomic Energy. Geneva (United Nations), 24, 255.

SAHASRABUDHE, M. B.—(1958) *Nature, Lond.*, **182**, 163.

Idem, NARURKAR, M. V., KOTNIS, L. B., TILAK, B. D. AND BHAVSAR, M. D.—(1959) *Ibid.*, **184**, 201.

Idem, NERURKAR, M. K., NARURKAR, M. V., TILAK, B. D. AND BHAVSAR, M. D.—(1960) *Brit. J. Cancer*, **14**, 547.

SANGER, C.—(1959) *Amer. J. Roentgenol.*, **81**, 498.

UMBREIT, W. W., BURRIS, R. H. AND STAUFFER, I. F.—(1951) 'Manometric techniques and tissue metabolism', Minneapolis (Burgess Pub. Co.), p. 15.

WARAVDEKAR, S. S. AND RANADIVE, K. J.—(1957) *J. nat. Cancer Inst.*, **18**, 555.

EXPERIMENTAL REGIONAL ADMINISTRATION (PERFUSION AND INFUSION) OF CHLORAMBUCIL (p-N,N-Di-(β-CHLOROETHYL) AMINOPHENYLBUTYRIC ACID)

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ISOLATED regional perfusion, introduced by Creech, Krementz, Ryan and Winblad (1958), in an attempt to reduce damage to the haemopoietic system during chemotherapy, may become a useful technique in the treatment of cancer.

This, and much of the later work, however, has been done on an empirical basis and it was decided to investigate the behaviour of an alkylating agent during the experimental perfusions of dogs to obtain some idea of the optimum conditions for clinical use. Factors such as temperature, pH and oxygenation can be altered in this isolated system and it is important to know which affect the behaviour of the drug and its reaction with normal tissue. The alkylating agents are not completely specific so that the difference between drug levels that will destroy normal and cancer tissue is small. If the activity of the drug is increased by modifying conditions then an otherwise safe dose may cause tissue damage.

Melphalan (L-p-di-(β-chloroethyl) aminophenylalanine) has a definite place in the treatment of melanomas and possibly sarcomas, but appears ineffective against adenocarcinomas (Creech, personal communication). Another drug might be more effective against the latter tumours and Chlorambucil was selected for these experimental perfusions since it had been used with some success against ovarian tumours at the Royal Marsden Hospital.

Chlorambucil was much more rapidly absorbed by tissue than Melphalan during the first 5–10 minutes of a perfusion. Thus it should be a better drug for regions of limited isolation, e.g. the pelvis, where unavoidable leaks into the systemic circulation result in complete mixing of the systemic and regional blood within 30–40 minutes.

In some experiments the main regional artery was occluded and blood containing Chlorambucil was pumped slowly through the artery below the occlusion to determine whether significant local concentration occurred. This is a development of the work of Barberio, Klopp, Ayres and Gross (1951) who investigated the arterial injection of HN2 (methyl di(β-chloroethyl) amine) Arterial infusion is a less severe procedure than perfusion and would enable regular repeated regional administration of suitably rapidly absorbed drugs. From radiotherapeutic experience, it might be expected that these " radiomimetic " drugs would be more effective when used several times.

MATERIALS AND METHODS

Estimation of Chlorambucil in blood.—This was carried out by a modification of the method described by Klatt, Griffin and Stehlin (1960).

Reagents.—0·25 M (isotonic) sucrose, 0·6 M pH 4·5 acetate buffer, 5 per cent w/v γ-(4-nitrobenzyl) pyridine in methylethylketone and 50 per cent v/v triethylamine (redistilled if not colourless) in acetone. The γ-(4-nitrobenzyl) pyridine was prepared from 4-benzylpyridine according to Bryans and Pyman (1929).

Procedure.—1 ml. of blood was added to 3 ml. of isotonic sucrose in a glass stoppered tube. After mixing by inversion the stopper was removed, the cells were separated by centrifugation and 3 ml. of the supernatant added to a second stoppered test-tube containing 0·5 ml. of the γ-(4-nitrobenzyl) pyridine solution and 0·1 ml. of the acetate buffer. This first part should be completed in 2–3 minutes, but the tubes may now be left overnight in the refrigerator if necessary. The tubes were heated in a water-bath at 80° C. for three hours in the case of Chlorambucil but this varied with other alkylating agents. The reaction was complete in 20 minutes with Melphalan and HN2. A flat metal lid was placed over the tubes while lowering them into the bath. This allowed the stoppers to rise slightly to release excess pressure, but prevented them from blowing out of the tubes. After cooling in ice-water the protein precipitate was removed by centrifugation and 2 ml. of the supernatant pipetted into a third set of tubes.

The absorbance of the solutions at 565 mμ. was read in a spectrophotometer 2–3 minutes after addition of 2 ml. of the triethylamine-acetone mixture. This was because there was an initial intense blue colour even in the blanks which very rapidly faded. The residual colour was unstable and was read within 5 minutes of the mixing with triethylamine.

A concentration of 100 μg. Chlorambucil/ml. blood gave an extinction of 0·6 in a 1 cm. cell. This was for blood containing heparin only ; citrate reduced the colour intensity probably by competing with the γ-(4-nitrobenzyl) pyridine for the alkylating agent. Heparin and papaverine in the concentrations normally used did not affect the colour development.

Labelling of erythrocytes with ^{51}Cr.—Cells were labelled by the method of Mollison and Veall (1955). A known quantity of labelled cells were added to the circuit at the same time as the alkylating agent. The cells spun down from the sucrose solution during the estimation of Chlorambucil were counted by placing the tubes in a scintillation counter well.

Surgical procedure

The experiments were carried out on male greyhounds weighing approximately 25 kg. General anaesthesia was induced by intravenous Nembutal 30 mg./kg. body weight. The dogs were positioned supine with extension of the lower limbs. The right groin was shaved and cleaned and prepared with 2 per cent solution of Hibitane in 70 per cent ethanol. The right femoral vessels were cannulated. All operations were carried out with antiseptic precautions.

Apparatus

1. Oxygenator—" De Wall " bubble type made from " Perspex " with a reservoir which contained 200 c.c. of blood.

2. Pump—Roller type as supplied by Watson Marlow Ltd.

3. Heat Exchanger—This consisted of a laboratory Pyrex ground glass coil condenser. The blood circulated through the helix and the outer jacket was warmed or cooled by water from a bath equipped with a thermostatically controlled heater and pump.

4. Tubing consisted of polyvinyl chloride (Portex) non-toxic of 4 mm. internal diameter with nylon connections to fit and nylon cannulae of 2 and 3 mm. external diameter.

Drugs used

1. *Chlorambucil.*—The free acid was dissolved in 0·1 N NaOH (about 2 ml. per 50 mg.). The solution was then mixed with 2–3 volumes of pH 7·4 propylene glycol buffer before addition to the perfusion circuit.

Buffer: 45 g. propylene glycol, 2 g. potassium dihydrogen phosphate (KH_2PO_4) adjusted to pH 7·4 with NaOH and made up to 100 ml. with distilled water.

2. *Melphalan.*—This was dissolved in the minimum amount of 0·1 N HCl and mixed with the propylene glycol buffer as for Chlorambucil.

3. *Heparin* (Evans Medical) "Pularin" 2 mg./kg.

4. *Polybrene* (Abbott) 1 mg. per 1 mg. of Heparin.

5. *Silicone* (Hopkins and Williams) Solutions of Anti-foam A.

6. *Nembutal* (Abbott) Pentobarbitone sodium 30 mg./kg.

Arrangement of perfusion circuit

The venous cannula was connected by tubing to the oxygenator, which was situated below the level of the dog and the blood drained by gravity to the oxygenator. From the reservoir of the oxygenator tubing led to the pump and from here to the heat exchanger and then to the arterial cannula. A thermometer was inserted into the arterial tube within 12 in. of the cannula. There was also a lead to a manometer and a by-pass tube connected the arterial to venous sides of circuit.

Vessel exposure

A 6 cm. axial incision in the right groin in the line of the femoral vessels was made and 4 cm. of the femoral vessels exposed. A medial circumflex branch of the artery which crossed the vein when present had to be ligated and divided. Two separate tapes were passed around both vein and artery. After heparinisation the vessels were occluded by traction in the tapes and then opened transversely with scissors and a cannular introduced into the vein. At this stage the circuit was primed with blood taken off the dog via this cannula—later the artery was cannulated.

The perfusion

Following cannulation a tourniquet consisting of $\frac{1}{4}$ in. red rubber tubing was run tightly twice around the upper thigh and clamped in position. Complete occlusion was difficult but possible in the majority of cases.

The isolated circulation was commenced by opening the venous line, then arterial; when satisfactory the by-pass clamped off. Temperature, pressure and

the level of blood in the reservoir were continuously recorded. The blood level rose rapidly if an inefficient tourniquet had occluded the veins but not the arteries. The tourniquet was adjusted until arterial occlusion was obtained as shown by a constant reservoir level.

After perfusion had proceeded satisfactorily for 10–20 minutes, the by-pass was opened and the arterial and venous tubes closed. The drug and labelled cells were then added to the reservoir and mixed in the by-pass circuit for five minutes. The circuit was then re-opened to the limb and the by-pass closed. Perfusion with the drug was continued, usually for 60 mniutes. Tissue temperature was taken by placing a thermometer deep in the quadriceps muscle.

At the end of the perfusion the cannulae was removed and the vessels double ligated above and below these openings. The collateral circulation was adequate to supply the necessary blood to the lower limb in the dog following femoral artery ligation. The incision was closed by interrupted silk sutures. Recovery from anaesthesia took 4–6 hours.

Regional infusion

The technique of regional infusion consisted of exposure of main vessels supplying the region followed by occlusion and cannulation of the artery as described above. The main venous return was not interfered with and venous puncture was performed for sampling only. The same apparatus was used for infusion as described above for regional perfusion, except that the venous cannula and return tube were not necessary.

In intra-arterial injections without occlusion there was no interference of the blood flow.

RESULTS

Observations on perfusions of the hind limb of a number of dogs indicated that physiological conditions for circulation were best simulated by a flow rate of 50–70 ml. with an arterial pressure of 100–160 mm./Hg. and temperature of 37·5° C. The flow rate increased with arterial pressure but wide ranges of flow rate were accommodated by the limb vascular reserve. Flow rates of 260–280 ml./minute were necessary to increase the pressure to 300 mm./Hg. If the blood was not oxygenated during perfusion the veins became distended and the limb blue, and later swelling and petechiae occurred.

With 97 per cent O_2 and 3 per cent CO_2 hind limb perfusions have been carried out for up to 120 minutes without any signs of tissue damage in the limb. Under these conditions the pH of the blood remained within normal limits (7·3–7·4). Normally only slight haemolysis occurred in the blood at end of the perfusion.

Perfusion with Chlorambucil

Dogs perfused with Chlorambucil can be divided into two groups, in which

(1) *No changes were noticed during perfusion.*

These perfusions were carried out at 37·5° C. and the concentration of Chlorambucil below 200 μg./ml. (Fig. 1). The arterial pressure remained constant at 100–120 mm./Hg., and flow rate 70 ml./minute. The Chlorambucil concen-

tration fell from 175 μg./ml. to 56 μg./ml. in 5–10 minutes and then remained almost constant for the rest of the perfusion; the venous and arterial concentrations were equal after 5 minutes. The fall in level of ^{51}Cr-tagged erythrocytes was due to dilution in the circulating fluid in the perfused limb.

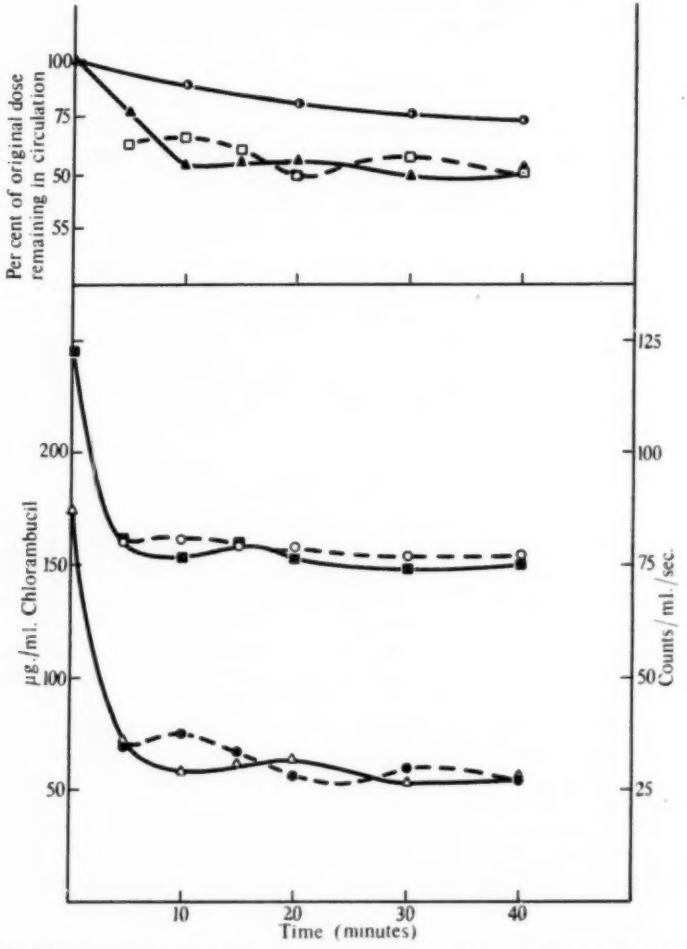

FIG. 1.—Hind limb perfusion without tissue necrosis at 37·5° C. Continuous lines indicate the level in the blood entering, broken lines in the blood leaving the limb; ■—■, ○—○, counts/ml./sec. (^{51}Cr), △—△, ●—● Chlorambucil concentration, ▲—▲, □—□ drug concentration corrected for dilution by the blood in the limb by assuming that the vascular distribution of the ^{51}Cr labelled cells and the Chlorambucil were identical. *In vitro* decay of Chlorambucil in dog blood at 37·5° C. ◑—◑ The rate of the reaction of the drug with the blood during the perfusion would have been less since the temperature of the blood in the reservoir of the oxygenator was approximately 30–32° C. Three perfusions gave very similar results. Total dose 60 mg. Chlorambucil.

(2) *Obvious tissue necrosis occurred.*

Changes during perfusions in which tissue damage occurred include an early sudden rise in the arterial pressure which was violent and sustained (Fig. 2). The skin became pink with the superficial veins contracted. There was a steady reduction in the venous return, the blood became more concentrated and dark coloured and did not change to a scarlet colour in the oxygenator. These changes

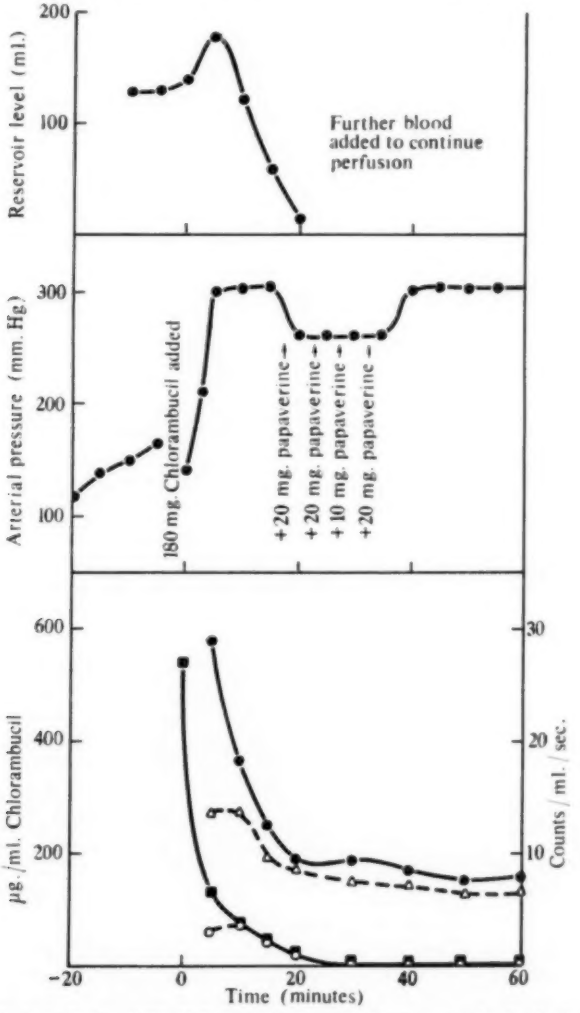

FIG. 2.—Hind limb perfusion at 37·5° C. resulting in rapid tissue necrosis. In the lowest section continuous lines indicate levels in the blood entering, broken lines leaving the limb; ●—●, △—△ counts/ml./sec. ■—■, ○—○, Chlorambucil concentration. Three perfusions gave similar results. Total dose 180 mg. Chlorambucil.

were progressive and were followed by severe swelling of the limb with increased local heat. The limb absorbed several times the circulating volume. These changes were probably caused by destruction of the capillary bed with loss of fluids from the vascular space. Immediate post-mortem examination of these

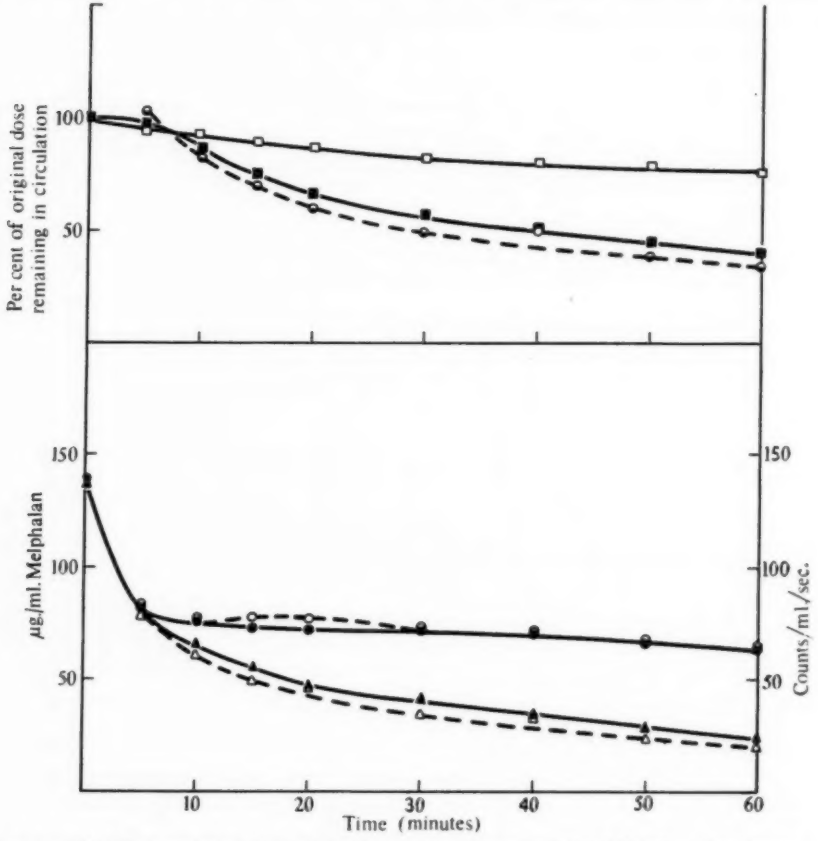

FIG. 3.—Hind limb perfusion at 37·5° C. without tissue necrosis using Melphalan. Continuous lines indicate levels in the blood entering, broken lines leaving the limb; ●—●, ○—○ counts/ml./sec. ▲—▲, △—△ Melphalan concentration, ■—■ ◒,—◒, results corrected for dilution by the blood in the limb, □—□ *in vitro* decay of Melphalan (see Fig. 1). Total dose 30 mg. Melphalan.

limbs showed that the muscles were black and swollen and on cutting a blood-stained fluid flowed out which permeated through the muscle sheaths. No immediate thrombosis of the main arteries or veins was found.

Isolated limb perfusions at 37·5° C. were carried out with low concentrations of Melphalan which produced no tissue damage under the conditions described above for Chlorambucil (Fig. 3).

Factors which affect the action of the drug

(1) *Concentration.*—In no experiment at 37·5° where the concentration of Chlorambucil was under 200 μg./ml. did tissue necrosis occur. Concentrations between 250–300 μg./ml. at 37·5° led to post-operative swelling of the perfused region from which recovery took place in 3–4 days. Higher concentrations led to immediate toxic changes during perfusions as previously described.

Perfusion with a total dose of 180 mg. of Chlorambucil in a 22 kg. dog at a concentration of 540 μg./ml. (Fig. 2) gave immediate destruction of the limb while a 23 kg. dog perfused with the same dose of Chlorambucil but with an increased circulatory volume giving a concentration of 275 μg./ml. had only mild post-operative oedema. In a 25 kg. dog use of 86 mg. of Chlorambucil at a concentration of 250 μg./ml. again led to only mild post-operative oedema of the perfused limb.

(2) *Temperature.*—The reaction velocity of Chlorambucil is doubled on raising the temperature 10°. That the reaction *in vivo* varies in a similar way is shown by the following experiments.

(*a*) A 22 kg. greyhound perfused with 180 mg. of Chlorambucil (concentration 540 μg./ml.) 37·5° C. suffered severe destruction of the limb in 10 minutes.

(*b*) A 25 kg. greyhound perfused with 130 mg. of Chlorambucil (concentration 400 μg./ml.) at 42° C. suffered even more rapid and complete necrosis.

On reducing the temperature of the arterial blood to 24° C. there was marked reduction in the drug uptake. At this temperature there was vascular spasm leading to reduction in capillary circulation.

(3) *Flow rate.*—Flow rates between 10–70 ml./minute were used and no gross difference in the rate of drug uptake occurred over this range.

(4) *Steroids.*—On two occasions during perfusion when signs of tissue damage were present 100 mg. of hydrocortisone was added to the perfusion fluid but nevertheless the limb progressed to necrosis.

(5) *Papaverine.*—This drug has a marked effect on arterial musculature, causing vascular dilatation and the opening up of arterio-venous shunts in the perfused region, thus reducing the circulation time. This effect might cause a partial by-pass of a tumour-bearing area and for this reason it seems inadvisable to use papaverine. The increase in arterial pressure which occurs on perfusing Chlorambucil occurs at a level of the drug which is toxic to the normal tissues and therefore should be avoided. Administration of papaverine when arterial pressure was high reduced this pressure as shown in Fig. 2, but did not prevent destruction of the tissues.

Regional infusions

All infusions were carried out at an arterial blood temperature of 37·5° C. In two experiments the technique of perfusion was carried out on the hind limb and the entire venous return was collected and not recirculated. Fresh blood containing Chlorambucil was twice added to the reservoir when the latter was nearly empty (Table I).

In all other infusions there was no occlusion of the venous return from the region. Chlorambucil and tagged erythrocytes were well mixed in the blood

TABLE I.—*Results of Attempts to Wash Out Chlorambucil that had been Absorbed by the Limb from the Extra-corporeal Circulation with Fresh Blood*

Number of dog	1	2
Dog's weight	23 kg.	23 kg.
Chlorambucil contained by blood entering the limb (200 ml. over 10 min.)	52 mg.	54 mg.
Immediate return contained	16 mg.	7·5 mg.
1st wash out contained 200 ml.	3 mg.	8·2 mg.
2nd wash out contained 200 ml.	1 mg.	—
Total Chlorambucil leaving limb	20 mg.	15·7 mg.

used for infusion. If the assumption was made that the vascular distribution of the Chlorambucil and the tagged cells remained identical the percentage of Chlorambucil leaving the region may be calculated from :

$$\frac{\text{Drug conc. in vein draining region} - \text{conc. in systemic circulation}}{\text{Drug conc. in the infused blood}} \times 100$$

$$\frac{\text{Counts/ml./sec. in vein draining region} - \text{counts/ml./sec. in systemic circulation}}{\text{Counts/ml./sec. in the infused blood}}$$

= per cent of total dose leaving the region.

Two right femoral infusions were carried out on dogs weighing 22 kg. and 31 kg. respectively. Samples of venous blood were taken from the right femoral vein at regular intervals throughout the infusion. 60 mg. of Chlorambucil was given in each infusion and no toxic changes occurred in either case. The results were similar and are presented in Fig. 4. These results show that the average Chlorambucil return in the venous blood from the infused hind limb is approximately 50 per cent.

Infusions of special regions

(1) *Pelvic infusion.*—The right external iliac artery was cannulated while the aorta below the region of the inferior mesenteric was clamped as was also the left external iliac in a dog weighing 11·5 kg. Venous samples were obtained from the inferior vena cava and 45 mg. of Chlorambucil was administered. The results (Fig. 5) show that approximately 50 per cent of the amount of drug infused returned. A similar result was obtained on repeating the experiment.

(2) *Infusion of the right side of brain, head and neck.*—The right carotid artery of a 29 kg. Labrador was cannulated and infused with 60 mg. of Chlorambucil in 250 ml. of blood. During the infusion there was a profuse secretion of mucus from the right nostril and the character of the respiration changed, but no vasomotor changes occurred. The dog made a complete recovery. The results (Fig. 6) show that 60 per cent of the Chlorambucil given returned from the region into the systemic circulation.

In experiments in which dogs were given intravenous whole body doses of 4 mg./kg. body weight, severe leucopaenia with weakness, vomiting and diarrhoea always resulted by the seventh day. Doses of 2 mg./kg. caused no changes in general health or blood picture in dogs. It may be deduced that in cases of infusion where there is no sign of toxicity and no marrow depression, the amount of venous leak is less than 2 mg./kg. : in cases of severe reaction then at least 4 mg./kg. has leaked into the general circulation.

The hind limbs of a 31 kg. Labrador were infused with a total dose of 120 mg. of Chlorambucil and resulted in no systemic ill effects, therefore the venous return would have been 60 mg. or less, i.e. 2 mg./kg., in agreement with the results shown in Fig. 4.

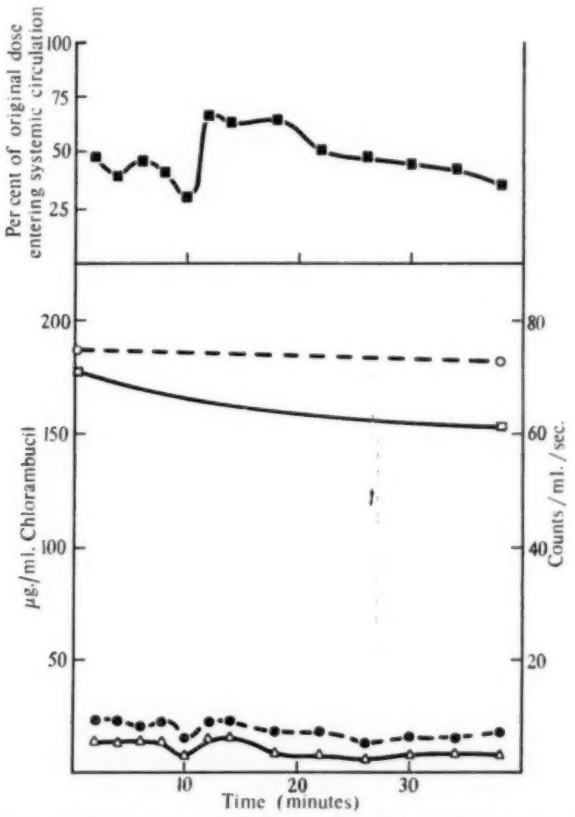

FIG. 4.—Hind limb infusion at 37·5° C. Broken lines indicate ^{51}Cr levels, continuous lines Chlorambucil concentrations; ○—○, □—□ blood entering femoral artery, ●—●, △—△ in samples taken from the femoral vein ■—■ see text. Systemic samples gave 0·9, 1·35, 2·0, 2·2 counts/ml./sec. and had drug concentrations of 2, 2·5, 1·5, 2 μg./ml. at 10, 20, 30 and 40 min. respectively. Total dose 60 mg. Chlorambucil.

In another experiment a 21 kg. greyhound was given 120 mg. of Chlorambucil in 400 ml. by infusion of the femoral vein; this led to evidence of mild systemic toxic signs—vomiting, loss of weight, depression in leucocytes and increased blood urea. From our findings of a 50 per cent venous return of Chlorambucil it may be estimated that this dog received into his systemic system 60 mg. (i.e. 50 per cent infused dose), which is 3 mg./kg., a dose which would be expected to give the above clinical picture.

DISCUSSION

In a perfusion using a therapeutic dose of Chlorambucil (Fig. 1) there was rapid initial drop in concentration after which the levels in the arterial and venous blood were identical but slowly declined.

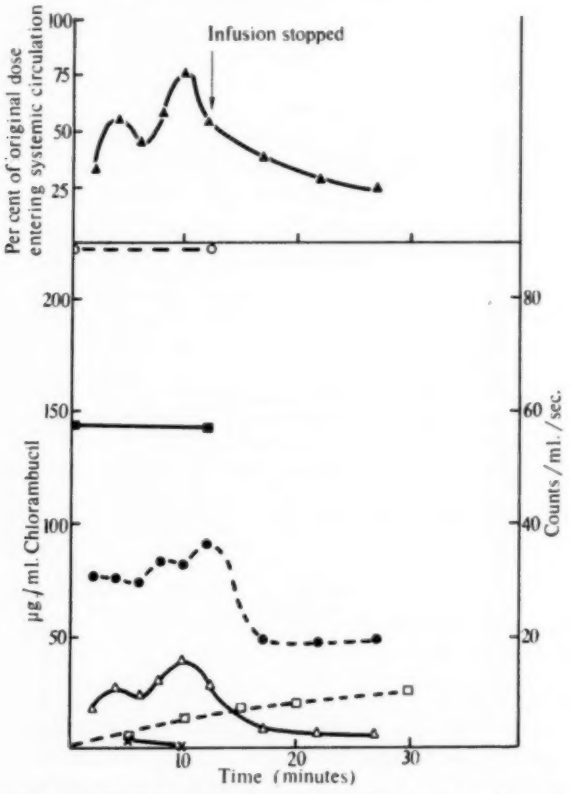

FIG. 5.—Pelvic infusion at 37·5° C. Broken lines indicate ^{51}Cr levels, continous lines Chlorambucil concentrations; ○—○, ■—■ blood entering right external iliac artery, ●—●, △—△ blood from inferior vena cava, □—□, ×—× systemic levels. The percentage of drug leaving the region was determined as described in the text. Total dose 60 mg. Chlorambucil.

Part of the initial drop is due to dilution of the drug by the blood in the limb, as shown by a similar smaller drop in the concentration of the ^{51}Cr-labelled erythrocytes. Complete mixing of the isolated circuit occurs in 5–10 minutes and allowing for the dilution 45–55 per cent of the Chlorambucil added to the reservoir has left or reacted in the vascular system during this time. *In vitro* experiments determining the rate at which Chlorambucil reacts in blood at 37·5° indicate that most of this is the result of absorption by the limb (Fig. 1).

When therapeutic doses are administered approximately 50 per cent of the

Chlorambucil leaves the blood in the first 5–10 minutes and since subsequently the arterial and venous lines are at the same concentration no more drug is absorbed by the limb. When destructive doses of Chlorambucil are used (Fig. 2) the drug level rapidly falls to undetectable levels. This may be due to breakdown of the capillary bed and subsequent uptake by the tissues.

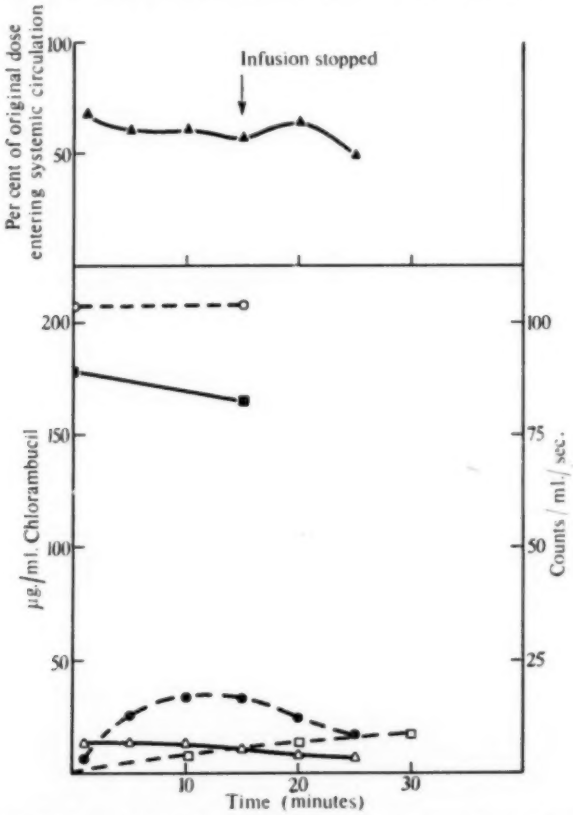

FIG. 6.—Infusion of right side of head and neck at 37·5° C. Broken lines indicate ^{51}Cr level, continuous lines Chlorambucil concentration; ○—○, ■—■ blood entering right carotid artery, ●—●, △—△ blood from right jugular veing, □—□ systemic ^{51}Cr. The drug entering the systemic circulation was rapidly absorbed from the vascular system since the Chlorambucil levels were too low to be detected. The percentage of drug leaving the region was estimated as described in the text. Total dose 60 mg. Chlorambucil.

The results given by a normal perfusion (Fig. 1) seem to indicate that equilibrium is rapidly attained between the limb tissue and the blood. The data in Table I, however, suggest that there may be some mechanism, either in the cells or the vascular supply which prevents further absorption after the first 10 minutes of perfusion. There is, therefore, little advantage in prolonging the perfusion for more than 10–15 minutes and indeed it would seem likely that the slow infusion

of Chlorambucil (up to twice the systemic dose at a concentration of approximately 200 μg./ml. at 37·5°) into the main artery supplying the tumour-containing region would be the best method of administration. Direct arterial injection in the normal manner is not considered as satisfactory because of the destructive effects of a concentrated solution, the comparison between the limb damage caused by direct injection of one femoral artery and infusion of the other in one of the dogs being marked. Furthermore, occlusion of the artery will slow the regional blood flow and aid the local absorption of the drug.

The rapid initial absorption of Chlorambucil is probably connected with the nature of the group attached to the di-β-chloroethyl part of the molecule since Melphalan in which L-phenylalanine replaces phenylbutyric acid is absorbed slowly but throughout the entire perfusion (Fig 3).

Several drugs are to be tested to determine the reason for the initial rapid absorption of Chlorambucil ; it may be due to the surface active properties of the drug. A knowledge of the chemical structures which will enable nitrogen mustards to penetrate tissues rapidly and to be held until reaction has occurred could be used to synthesise the alkylating agents most suitable for regional use.

SUMMARY

The behaviour of Chlorambucil in experimental regional perfusions and infusions in the limbs of dogs has been studied in order to determine the probable optimum conditions for clinical use.

At 37·5° C. concentrations below 200 μg./ml. cause no damage. Levels between 250–300 μg./ml. cause mild reversible oedema, but with higher levels than this necrosis occurs. Increasing the temperature increases the activity of the drug.

The initial rapid tissue uptake of the drug in comparison with Melphalan is shown. Evidence is presented in support of use of Chlorambucil by intra-arterial infusion.

We wish to express our gratitude to the Council of the Royal College of Surgeons of England for permission to work at the Buckston-Browne Research Farm, and Mr. F. Watson for skilled technical help. Dr. E. Field kindly provided the labelled cells and Dr. W. Ross the sample of Chlorambucil.

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REFERENCES

Barberio, J. R., Klopp, C. T., Ayres, W. W. and Gross, H. A.—(1951) *Cancer*, **4**, 1341.
Bryans, F. and Pyman, F. L.—(1929) *J. chem. Soc.*, p. 549.
Creech, O., Krementz, E. T., Ryan, R. F. and Winblad, J. N.—(1958) *Ann. Surg.*, **148**, 616.
Klatt, O., Griffin, A. C. and Stehlin, J. S.—(1960) *Proc. Soc. exp. Biol. N.Y.*, **104**, 629.
Mollison, P. L. and Veall, N.—(1955) *Brit. J. Haemat.*, **1**, 62.

A CHOLANGIOCELLULAR CARCINOMA OF THE FOWL (GALLUS DOMESTICUS)

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Bile duct carcinomata appear to be among the rarer tumours of the domestic fowl. A single example has been recorded by Schuchmann (1931), two by Heim (1931), three by Bullis and Olson (1943), and one by Campbell (1945). It is difficult to assess the incidence ; the three cases seen by Bullis and Olson (1943) occurred among 301 avian neoplasms in their collection, while Campbell's (1945) case was found in a study of 386 tumours. On the other hand, neither Eber and Malke (1932), nor Goss (1940), specifically mention bile duct tumours in their respective surveys of 371 and 1445 avian neoplasia.

Neither the gross nor the microscopic appearance of this tumour of the fowl has been recorded, except by Campbell (1947) who briefly described it as multiple green tumours scattered through the liver. Histologically, the relationship to bile duct epithelium was said to be evident.

REPORT OF A CASE

The tumour occurred in a female Brown Leghorn from the inbred flock at this Centre. The bird died at the age of 29 months without a history of illness, although the bodily condition was poor and few eggs had been laid during the previous nine months.

Examination post mortem

The cadaver was emaciated. Moderate numbers of white nodules of up to 0·5 cm. diameter were present on the surface and in the substance of the slightly enlarged liver. A few small cyst-like structures, some bright green and some bright red, were embedded in the surface of the liver, while projecting from it were several small, red, multilocular cysts, the largest being 1·5 cm. in diameter and resembling a raspberry.

There were hard, white nodules on the peritoneum, which was slightly thickened. Considerable scirrhous involvement of the pancreas and wall of the duodenum had occurred. The ovary contained several pea-sized white nodules, and there were three grey, pediculated cystic follicles, each about 3 cm. in diameter, attached to it.

Microscopic examination

Proliferations of neoplastic bile ducts had occurred throughout the liver (Fig. 1). Short oval or circular ducts invaded nearly every lobule. Their

component cells were cuboidal with pale cytoplasm, a large round vesicular nucleus and one, or more rarely, two, nucleoli (Fig. 2). The nucleus to plasma ratio was less than in the hepatic cells. Mitoses were rare. Invasion by the neoplastic alveoli was intersinusoidal or indiscriminate, and accompanied by necrosis of adjacent liver cells (Fig. 2). There was a generalized slight distension of the spaces of Dissé. Mild fibrosis occurred in the region of the new growths.

The tumour sometimes formed large cystic spaces lined by cuboidal, columnar or, less frequently, flattened bile duct epithelium (Fig. 3). Occasionally this epithelium projected into the lumen, but pronounced papilliferous structures were not seen. When the cells were columnar, their nuclei were oval and proximally situated. The cysts were surrounded by a dense fibrous tissue matrix (Fig. 3) infiltrated by foci of heterophils, plasma cells, and haemosiderin-laden macrophages.

Within the lumina of the cysts was a little eosinophilic amorphous material, a few desquamated lining cells, and an occasional lymphocyte or macrophage. Frequently the cysts contained large numbers of intact erythrocytes (Fig. 3, 4). Cysts occurred in the liver parenchyma and also projecting from its surface (Fig. 4); the presence of fresh blood in multilocular examples of the latter type imparted a pink, raspberry like appearance in the macroscopic specimen.

Apart from fibrosis around the neoplastic growths, the liver as a whole was not cirrhotic. One gained the impression that the connective tissue, certainly around the small proliferating bile ducts, was laid down in response to the tumour, rather than preceding it.

Metastases of alveoli composed of cells similar to those of the liver tumour were present on the peritoneum, splenic capsule, pancreas, duodenum, oviduct and ovary. They were accompanied by considerable fibrosis.

DISCUSSION

Because of the occurrence of mixed types of primary liver carcinoma and the similar embryogenesis of liver and intrahepatic bile duct cells, Berman (1951) says that rigid classification into hepatic or bile duct carcinoma of the human subject is efficient only where the tumour is uniform. This distinction is even more difficult in the fowl where tubule formation by an embryonic type of liver cells, closely resembling hyperplastic bile ducts, is a common response to liver damage (Campbell, 1955–56; Siller and Ostler, 1961). However, the present tumour was well differentiated, and the appearance and arrangement of the

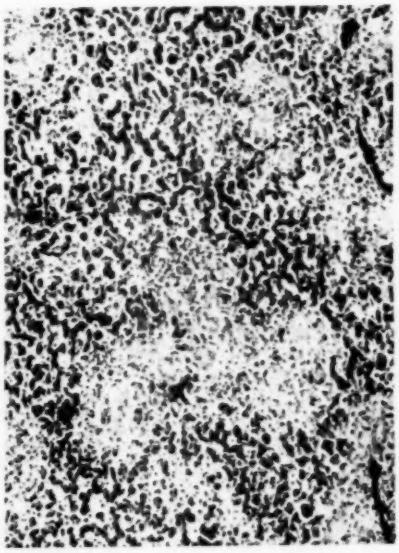
EXPLANATION OF PLATE

FIG. 1.—Proliferation of neoplastic bile duct alveoli throughout the liver. H. and E. ×100.

FIG. 2.—Necrosis of hepatic cells adjacent to an invading group of intersinusoidal bile duct cells. H. and E. ×400.

FIG. 3.—Cystic alveoli in a fibrous matrix. The cysts are lined by cuboidal, columnar or flattened bile duct epithelium. In some lumina are large numbers of red blood corpuscles, and in others a few macrophages and cell debris. H. and E. ×60.

FIG. 4.—Extrahepatic multilocular cystic structure. The cysts are lined by neoplastic bile duct epithelium which is sometimes papilliferous, and most of their lumina contain blood. H. and E. ×40.



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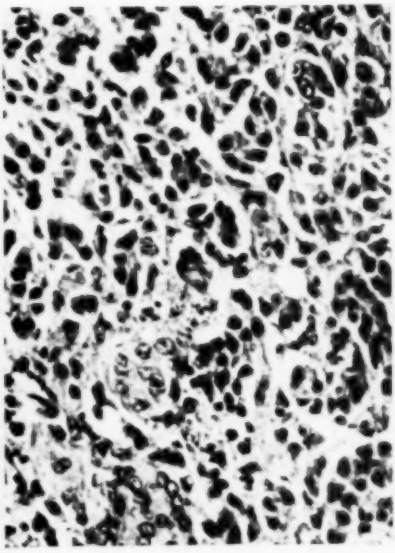

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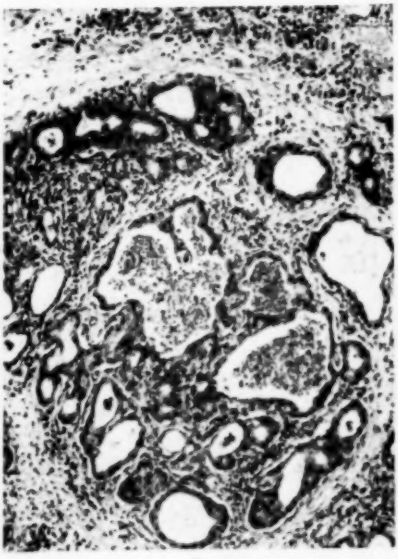

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cells closely resembled the human examples of cholangiocellular carcinoma described by Berman (1951). It adequately fulfilled the criteria suggested by Edmondson and Steiner (1954) for distinguishing carcinomata of the bile duct from those of the liver cell; the cells were of the bile duct type, they formed tubules rather than trabeculi, and the stroma was fibrous rather than capillary.

As is well known (Jackson, 1936; Berman, 1951; Cotchin, 1956), a degree of species specificity exists with regard to the type of liver tumour; hepatic cell carcinomata predominate in man, bovines and sheep while bile duct carcinomata are more common in carnivores. In 22 ducks affected with liver tumours, perhaps of genetic aetiology, Campbell (1949) found 17 hepatocellular, 1 cholangiocellular and 4 mixed types of carcinomata. Literature which adequately differentiates one or the other type of spontaneous tumour of the fowl is scanty; ten hepatocellular carcinomata appear to have been recorded (Jöest and Ernesti, 1916 (2); Heim, 1931 (2); Feldman, 1932 (1); Jackson, 1936 (1); Norris, 1936 (1); Bullis and Olson, 1943 (2); Campbell, 1947 (1)). Against this, seven bile duct carcinomata, as noted in the introduction to this paper, have been recorded. The present case is the sole primary liver carcinoma seen during the five years in which accurate records have been kept of a total of 1416 autopsies performed on this flock. Under these circumstances it is impossible to assess the relative incidence of the two types of carcinomata in the fowl.

The occurrence of blood filled cysts is uncommon in cholangiocellular carcinomata; they are mentioned neither by Berman (1951) nor by Edmondson and Steiner (1954), although the latter say that haemorrhage is more common in liver cell carcinomata. In domestic animals, Feldman (1932) recorded carcinomata of the specialized epithelial cells of the livers of dogs, cattle and sheep with extravasations of blood directly contacting the tumour cells. Jackson (1936) described this phenomenon in a dog, and termed it pseudo-haemangiomatoid cholangiocellular carcinoma. Although bile duct carcinomata are said to use the sinusoidal endothelium as a basement membrane (Edmondson and Steiner, 1954), there was no evidence of an endothelial lining to the cysts in the present case, where the blood directly contacted the tumour cells. As in Jackson's (1936) case, the tumour cannot therefore be described as a true haemangioma.

Metastases are to be expected following so intimate an association with the blood stream. In man and other animals, metastases are common in the lungs, but, as in the present case, these are not an invariable site.

Alkaloids of *Senecio jacobea* have been shown experimentally to be capable of eliciting liver tumours, including cholangiocellular carcinomata, in the fowl (Campbell, 1955–56), and in mammals associations have been suggested between the tumours and malnutrition, race, toxins, and cirrhosis due to various causes (Berman, 1951). In the present case there was no evidence of toxins, malnutrition, genetic factors, environmental abnormalities, or preceding cirrhosis, for the husbandry and diet had been similar to that of the rest of the inbred flock at the Centre.

CONCLUSIONS

A cholangiocellular carcinoma occurring in a domestic fowl is described, and the structure, incidence and aetiological factors associated with this type of tumour discussed in comparison with similar neoplasia of mammals.

UMI

REFERENCES

BERMAN, C.—(1951) ' Primary Carcinoma of the Liver '. London (H. K. Lewis and Co. Ltd.).
BULLIS, K. L. AND OLSON, C.—(1943) *Amer. J. vet. Res.*, **4**, 382.
CAMPBELL, J. G.—(1945) *J. comp. Path.*, **55**, 308.—(1947) ' Neoplastic Diseases in the Fowl ' *in* ' Diseases of Poultry,' ed. by Blount, W. P. London (Baillière, Tindall and Cox).—(1949) *Brit. J.Cancer*, **3**, 198.—(1955–56) *Proc. roy. Soc. Edinb.*, B, **66**, 111.
COTCHIN, E.—(1956) ' Neoplasms of the Domesticated Mammals, a Review ". Farnham Royal, Bucks (Commonwealth Agricultural Bureau).
EBER, A. AND MALKE, E.—(1932) *Z. Krebsforsch*, **36**, 178.
EDMONDSON, H. A. AND STEINER, P. E.—(1954) *Cancer*, **7**, 462.
FELDMAN, W. H.—(1932) ' Neoplasms of Domesticated Animals '. London (W. B. Saunders).
GOSS, L. J.—(1940) *Cornell Vet.*, **30**, 75.
HEIM, F.—(1931) *Z. Krebsforsch.*, **33**, 76.
JACKSON, C.—(1936) *Onderstepoort J. vet. Sci.*, **6**, 3.
JÖEST, E. AND ERNESTI, S.—(1916) *Z. Krebsforsch.*, **15**, 1.
NORRIS, J. C.—(1936) *Amer. J. Cancer*, **26**, 778.
SCHUCHMANN, K.—(1931) *Arch. Tierheilk*, **63**, 154.
SILLER, W. G. AND OSTLER, D. C.—(1961) *Vet. Rec.*, **73**, 134.

TUMOURS OF MANY SITES INDUCED BY INJECTION OF CHEMICAL CARCINOGENS INTO NEWBORN MICE. A SENSITIVE TEST FOR CARCINOGENESIS: THE IMPLICATIONS FOR CERTAIN IMMUNOLOGICAL THEORIES.

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PIETRA, SPENCER AND SHUBIK (1959) reported the induction of malignant lymphoma (lymphocytic leukaemia) and lung adenomas in Swiss mice injected subcutaneously when new-born (less than 24-hour old), with 30 μg. 9,10-dimethyl-1,2-benzanthracene (DMBA) suspended in 1 per cent aqueous gelatine. Later, Stich (1960) obtained a similar result using a 60 μg. dose of DMBA.

We were interested in the report of Pietra *et al.* (1959) for two reasons. Firstly we were anxious to discover whether cell-free filtrates prepared from malignant lymphoma induced by the injection of a carcinogen could themselves be passed to other mice by means of cell-free filtrates (e.g. Gross, 1957), and this method of chemical induction of lymphoma seemed very suitable. Secondly, the method described by Pietra *et al.* (1959) offered the possibility of a highly sensitive test for carcinogenic activity. In the latter connection it was important to find out whether other strains of mice would respond in the same way as the Swiss mice used by Pietra *et al.*, and also whether leukaemia and lung tumours could be induced by the injection of other carcinogens, both strong and weak, into new-born mice.

At the time that the present paper was ready for submission a second paper by Pietra and his colleagues (Pietra, Rappaport and Shubik, 1961) was published. The latter extended their earlier findings in four directions; firstly they confirmed their original observation with DMBA; secondly they tested 4 other carcinogens, 3,4-benzopyrene, 20-methylcholanthrene, 1,2:5,6-dibenzanthracene, and urethane by the same method; thirdly they compared the subcutaneous and intraperitoneal routes of administration for each of the 5 carcinogens; and fourthly they reported the induction of tumours at sites other than the lung and the lymphatic system. Even more recently Fiore-Donati *et al.* (1961) have recorded similar findings in an experiment in which new-born mice were injected with urethane; and Kelly and O'Gara (1961) have obtained similar results with 20-methylcholanthrene and 1,2:5,6-dibenzanthracene.

Work reported in the present paper confirms and extends the studies of Pietra and his colleagues. Two inbred strains of mice were used, and their response to DMBA compared. Induction of tumours of many sites, including those mentioned by Pietra *et al.* (1961), is described. The testing of cell-free filtrates prepared from

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carcinogen-induced malignant lymphoma will be the subject of a later communication.

MATERIAL AND METHODS

Mice.—" CBA " and " 101 " inbred strains, both obtained originally from the MRC Radiobiological Research Unit, Harwell, were used (for further details see Snell *et al.*, 1960). Metal cages were used throughout the experiment. Diet 41B and water were provided *ad libitum*.

Chemicals.—9,10-dimethyl-1,2-benzanthracene (DMBA) was obtained from L. Light & Co. ; gelatine from British Drug Houses ; and croton oil from Stafford Allen and Co.

Preparation and administration of DMBA/gelatine suspension.—The methods of preparation and administration described by Pietra, Spencer, and Shubik (1959) were followed precisely. Thirty μg. DMBA suspended in 15 μl. of 1 per cent aqueous gelatine were injected subcutaneously in the interscapular region into test groups of both strains. One per cent aqueous gelatine alone was injected into a control group of the CBA strain. Untreated mice of both strains were also kept as controls. The general layout of the experiment is shown in Table I.

Litters which averaged 5–6 mice each were allotted randomly to test and control series, until groups of the required size were formed (7 to 11 litters per group —see Table I).

All mice were less than 24 hours old when injected.

Subsequent conduct of the experiments.—Litters were housed separately until weaning, at which time the mice were numbered on the ears and re-housed in boxes of 8 to 10 according to group and sex. A proportion of the mice failed to survive until weaning (Table I), but it was not possible to examine post mortem any of those which died.

After re-housing the mice were carefully examined, at first once weekly and, from the 10th week onwards, twice weekly until they were 1 year old, for evidence of malignant lymphoma, tumours of other sites, and other lesions. Sick mice were killed and examined post mortem, and at the end of the year all the survivors were killed and similarly examined.

Routine post mortem examination included a close scrutiny of the stomach wall after fixation by dilatation with formal saline.

RESULTS

In both strains, groups injected with DMBA survived less well, and had a higher incidence of malignant lymphoma, lung tumours, skin tumours, tumours of the fore-stomach, and tumours of other sites, than uninjected mice or mice injected with suspending medium only. These differences are considered below under separate headings.

Survival

It is doubtful whether treatment with DMBA had any unfavourable effect on survival during the first month of life (see Column 6 in Table I). The overall higher death rate during this period in CBAs as compared with " 101 "s, was probably due to the higher incidence of litter-eating in the former. This was common in our CBA colony at the time that the experiment was begun.

TABLE I.—*Effect of the Subcutaneous Injection of 30 μg. DMBA into New-born CBA and "101"-strain Mice: Survival and Incidence of Malignant Lymphomata*

Group	Strain	Treatment	Number of litters	Mice alive at 1 day	Deaths during first month	Deaths between 1 month and 1 year	Causes of death between 1 month and 1 year: Malignant lymphoma	Other neoplasms	Other causes	Not examined post mortem*	Mice alive at 1 year
1	CBA	DMBA in aqueous gelatine	8	44	5†	18	6	2	3	7	21
2	,,	Aqueous gelatine	7	44	17†	1	0	0	1	0	26
3	,,	None	10	49	10†	0	0	0	0	0	39
4	101	DMBA in aqueous gelatine	11	57	12	19	9	2	3	5	26
5	,,	None	9	47	3	4	0	0	1	3	40
				241	47	42		27		15	152

N.B.—

† The large numbers of deaths in CBA mice (Groups 1–3) during the first month is not a peculiar feature of the present experiment: eating of litters by mothers was common in our CBA colony at the time.

* Because of advanced decomposition.

Deaths between the end of the first month and one year were far more numerous in the DMBA-treated groups of both strains than in the control groups (for details see column 7 in Table I). The commonest causes of death during this period were malignant lymphoma of the stem-cell type (Pietra *et al.*, 1961) involving the thymus, and tumours of the lung. Six DMBA-treated CBAs, and 9 DMBA-treated " 101 "s died from lymphoma, and 2 mice of each strain from multiple, large, and/or malignant, lung tumours. Three DMBA-treated CBAs and 1 DMBA-treated " 101 " died without tumours of any site. No tumours were seen in either of 2 control mice (one from Group 2, and one from Group 5). No post mortem examination was possible in 15 mice (Table I). Combining the results from the two strains: 37/84 DMBA-treated mice died between 1 month and 1 year, whereas only 5/110 untreated or control-injected mice did so.

Malignant lymphoma with thymic involvement

The earliest clearcut difference between the DMBA-treated and the control groups was in the incidence of malignant lymphoma. As shown in Tables I and II a total of 15 cases were seen in the two groups treated with DMBA. Only one very late case was seen in the control groups. This was in an untreated " 101 "

TABLE II.—*Malignant Lymphoma Arising in Mice Injected when New-born with 30 µg. DMBA*

DMBA-treated CBA mice (Group 1)

Litter	Mice alive at 1 month ♀	Mice alive at 1 month ♂	Number which developed malignant lymphoma ♀	Number which developed malignant lymphoma ♂	Induction time (weeks)
A	3	3	1	—	29
B	3	0	—	—	
C	4	0	—	—	
D	4	2	2	—	22, 22
E	6	0	1	—	32
F	1	4	1	1	29, 22
G	4	1	—	—	
H	3	1	—	—	
	28	11	5	1	Av. = 26·0 weeks
	39		6 (15·4%)		

DMBA-treated " 101 "-strain Mice (Group 4)

	♀	♂	♀	♂	
I	1	3	—	2	10, 13
J	4	3	—	2	16, 20
K	0	5	—	—	
L	6	6	—	—	
M	0	2	—	2	13, 15
N	2	2	—	—	
O	1	3	—	1	25
P	2	4	—	—	
Q	5	3	2	—	13, 23
R	2	3	—	—	
S	0	0	—	—	
	17	28	2	7	Av. = 16·5 weeks
	45		9 (20%)		

mouse, and was discovered at routine post mortem at one year. It was of a different histological type from the other cases (*vide infra*). Table II shows that the cases of malignant lymphoma were distributed randomly between the DMBA-treated litters. It is doubtful whether there was a real sex difference in incidence in either strain ; in fact, the apparent differences were in the opposite direction in the two strains. The total incidence was 20 per cent of " 101 " mice, and 15·4 per cent of CBA mice, alive at 1 month, but this slight difference was associated with a striking difference in average induction time ; 16·5 weeks in " 101 "s, and 26 weeks in CBAs. The latter difference was analysed by the " t " test and found significant ($P < 0{\cdot}01$). This analysis, however, did not take into account the possibility that some of the DMBA-treated mice which died without post mortem may have had malignant lymphoma. This is unlikely, because they were not thought to have thymic tumours or enlarged lymph nodes when examined a few days before death, and because in both strains the average time of death of the

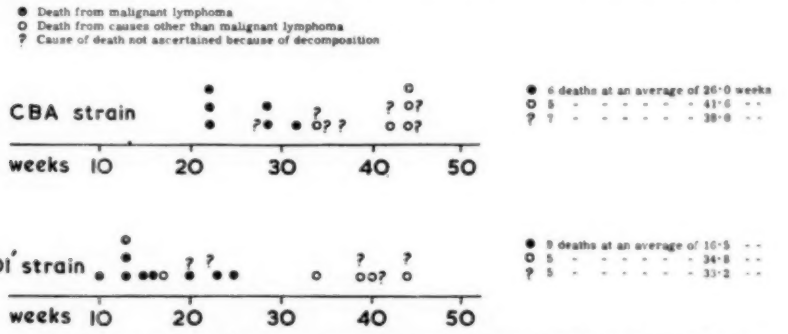

● Death from malignant lymphoma
○ Death from causes other than malignant lymphoma
? Cause of death not ascertained because of decomposition
CBA strain
weeks 10 20 30 40 50
● 6 deaths at an average of 26·0 weeks
○ 5 - - - - - - 41·6 - -
? 7 - - - - - - 38·6 - -
'101' strain
weeks 10 20 30 40 50
● 9 deaths at an average of 16·5 - -
○ 5 - - - - - - 34·8 - -
? 5 - - - - - - 33·2 - -

FIG. 1.—Comparison of induction-time of malignant lymphoma in CBA and " 101 "-strain mice after the injection of 30 μg. DMBA when newly born. This figure shows that deaths from unascertained causes occurred, for the most part, after the period during which malignant lymphoma appeared.

unexamined mice was considerably longer than that of the mice with lymphoma, and similar to that of mice dying without it (Fig. 1).

All the cases of malignant lymphoma which arose in the DMBA treated mice were of the histological type referred to by Pietra *et al.* (1961) as " stem cell ". In all of them there was gross enlargement of the thymus, so that it filled more than half the thoracic cavity, and an accompanying enlargement of some or all the lymph glands (axillary, inguinal, cervical, lumbar, pararenal, mediastinal, etc.). The spleen, on the other hand, was usually of normal size, and the liver always so.

The malignant lymphoma seen in the control untreated " 101 " mouse had a different distribution throughout the lymphatic system, and appeared quite different microscopically. There was slight enlargement of the left lobe of the thymus and more marked enlargement of the left pararenal lymph gland. The mesenteric lymph gland and spleen were also moderately enlarged, but the lymph glands in the neck, axillae, inguinal, and lumbar regions were of normal size. Microscopically the enlarged glands and thymus consisted predominantly of histiocytes with abundant eosinophilic cytoplasm. Mitotic figures were plentiful. The disease in this mouse was considered to be of the type referred to by Pietra

et al. (1961) as "histiocytic". No cases of lymphoma of the "lymphocytic" type, as described by Pietra and his colleagues, were seen.

Lung tumours

In both strains, mice treated with DMBA developed multiple benign, and occasional malignant, tumours of the lungs. By comparison, untreated mice of both strains and gelatine-injected CBAs developed a negligible number of such tumours, none of them malignant.

Pulmonary tumours were certainly present in several of the mice dying early from malignant lymphoma. However these were not counted because of the difficulty of distinguishing, except by microscopic examination, between adenomas and metastatic lymphomatous deposits. Apart from these, 4/10 DMBA-treated mice dying before they were 1 year old, had lung tumours.

TABLE III.—*Incidence of Lung Tumours 52 Weeks After Injection of 30 μg. DMBA into New-born CBA and "101"-strain Mice*

Group	Strain	Treatment	Mice alive at 52 weeks*	Mice with lung tumours/mice examined	Total tumours	Average lung tumours per mouse examined	Average diameter of largest tumour (mm.)
1	CBA	DMBA/Gelatine	21	16/18	222†	12·2	1·8
2	..	Gelatine	26	0/25	0	—	—
3	..	None	39	3/39	6	0·16	1·2
4	101	DMBA/Gelatine	26	24/25	327†	13·1	3·4
5	..	None	40	6‡/40	10‡	0·25	1·3

* Three mice from Group 1, and one each from Groups 2 and 4 died during the 52nd week and were too decomposed when found for post mortem examination.

† One malignant adenocarcinoma.

‡ Five of these mice, bearing 9 of the 10 tumours come from two litters and were housed together from weaning till death. It is possible that contamination with a carcinogen was responsible.

Table III shows the incidence of lung tumours in mice which survived for one year and were then killed. The DMBA-treated mice of both strains had an average of 12–13 tumours per mouse, whereas the incidence in the control groups was less than 0·3 tumours per mouse. The strains appeared to differ in their response to DMBA in that the average size of the largest tumours in the "101" mice was almost double that of the largest tumour in CBA mice. This difference was analysed by the "t" test and found to be significant ($P < 0{\cdot}01$). In fact not only the largest, but all the lung tumours in "101" mice tended to be larger than those in CBAs, and there can be no doubt that if records of the sizes of all tumours were available for comparison the difference would be even more significant.

Histologically all the tumours were of the well-known adenomatous type. Two of the tumours, one in a CBA mouse and one in a "101", were adherent to the chest wall and had metastased to the mediastinum and upper surface of the diaphragm. Apart from these undoubtedly malignant tumours there were others which showed microscopic evidence of infiltration of surrounding pulmonary tissue.

There was no sex difference in the lung tumour response of "101"-strain mice to treatment with DMBA: 16 males which survived the year had an average of

13·2 tumours per mouse, against an average of 12·8 in 9 females. No comparison was possible in the case of CBAs since only 2 males of Group 1 came to post mortem at 52 weeks.

Tumours of the skin

Benign papillomas and sebaceous adenomas of the skin were seen in 1 out of 18 CBA and in 7 out of 25 " 101 " DMBA-treated mice which were examined post mortem at 52 weeks. All arose between the 35th and 52nd weeks, and the majority were on the head and face. One of the sebaceous adenomas is shown in Fig. 2. No skin tumours were seen in 104 control mice examined at the same time. No malignant skin tumours were seen in any group.

Tumours and telangiectasia of the forestomach

Close examination of the stomach was not at first included in the routine post mortem examination. However, when the first batch of mice from Group 4 (DMBA-treated " 101 "s) were killed and examined, papillomas and bright red patches were seen in the wall of the forestomach. Thereafter all stomachs were carefully examined, after distension with fixative. Only tumours of 1 mm. diameter and over were counted, and special care was taken in interpreting irregular projections in the vicinity of the ridge between forestomach and glandular stomach. The results are shown in Table IV.

TABLE IV.—*Papillomas and Telangiectases in the Forestomachs of 52-week old CBA and " 101 "-strain Mice Injected when New-born with 30 µg. DMBA*

Group	Strain	Treatment	Number of mice examined for lesions of fore-stomach	Mice with papillomas of fore-stomach	Total papillomas	Papillomas 2 mm. diameter or more	Mice with telangiec-tases	Total telangiec-tases
1	CBA	DMBA/Gelatine	22	2	2	1	2	5
2	..	Gelatine	23	0	0	0	0	0
3	..	None	38	0	0	0	1	1
4	101	DMBA/Gelatine	20	16	64	7	6	15
5	..	None	30	0	0	0	0	0

Papillomas were seen in DMBA-treated mice of both strains but not in any of the control groups. The incidence was far higher in the " 101 " than in the CBA strain. Most of the papillomas were about 1 mm. diameter but a few exceeded 2 mm. diameter (Fig. 3). There were no malignant tumours.

The occurrence of bright red patches in the wall of the forestomach has not to our knowledge been reported previously. They were seen in both strains (Table IV). The majority were circular and some were as large as 2·5 mm. diameter. Six of these lesions were examined microscopically: they were found to consist of groups of dilated capillaries, in fact typical *telangiectases* (Fig. 4). In several of the mice which had these lesions, and in some that did not, there were macroscopically similar lesions in the proximity of one or more of the Peyer's patches of the small intestine. Unfortunately no precise records were made of the latter in the present experiment. However, vascular lesions of the gut wall are not uncommon in mice treated with DMBA or urethane as young adults (unpublished

data). Microscopic examination of the latter has shown them to be either telangiectases, or haemorrhages possibly derived from pre-existing telangiectases.

Hepatic tumours

Three mice in Group 1, 2 in Group 3, and 1 in each of the other 3 groups had parenchymal-cell liver tumours. Clearly this difference in incidence between the DMBA-treated and control groups was minimal. However it was interesting that the three CBA mice of group 1 had multiple (more than 10) tumours each, whereas the 3 control CBAs from Groups 2 and 3 had 2, 1 and 1 tumours respectively. Similarly, the mouse in Group 4 with liver tumours had 4 of them, whereas the mouse from Group 5 had only 1.

Histologically, several of the tumours showed cytoplasmic inclusions similar to those described by Burns and Schenken (1940) and Craigie (1955). In view of the sex difference observed by these authors in C_3H mice, it is of interest that 7 out of the 8 mice reported here were males. The remaining mouse, a gelatine-injected control of Group 2, was a virgin female, exactly 1 year old when killed. The tumour in this mouse was 5 mm. in diameter and of typical gross appearance : it was not examined histologically.

Tumours of other sites

Eleven mice, all of which had been treated with DMBA, had tumours of other sites. These are listed in Table V, and four of them are illustrated in Fig. 5–8.

Since only one or two examples of each kind of tumour were seen it is not proposed to describe them in detail.

Papillonephritis

Naked eye evidence of this disease, consisting of swelling, pallor, or calcification of the renal papilla, and cicatrisation of the renal cortex, was observed in 9 out of 26 DMBA-treated " 101 "-strain mice, and in 20 out of 39 untreated control mice of the same strain, killed when 1 year old. No cases of the disease were seen in CBA mice.

EXPLANATION OF PLATES

Lesions seen in mice injected when less than 24 hours old with 30 µg. DMBA in 1 per cent aqueous gelatine, and killed when 52 weeks old. Stained H. and E.

FIG. 2.—Sebaceous adenoma from skin of abdominal wall of a " 101 " ♂. ×48.

FIG. 3.—Papilloma of wall of forestomach of a CBA ♀. ×35.

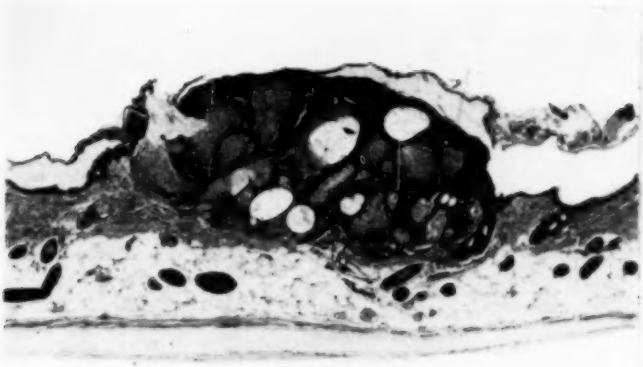
FIG. 4.—Telangiectasis in wall of forestomach of a CBA ♀. A group of grossly dilated capillaries are seen beneath the keratinized squamous epithelium. The wide gap between the dilated vessels and the muscle layers is an artifact. ×70.

FIG. 5.—Granulosa-cell tumour of ovary from a CBA. ×370.

FIG. 6.—Cortical adnoma in the kidney of a " 101 " ♂. ×65.

FIG. 7.—Malignant haemangioma infiltrating subcutaneous and muscle layers of thigh and abdominal wall of " 101 " ♀. Remnants of voluntary muscle are seen. Parts of this tumour were more cellular and mitotically active. ×330.

FIG. 8.—Tumour from parotid gland of a " 101 " ♂. Opinion was divided as to the nature of this tumour. The absence of cross-striations in a P.T.A. stain failed to support a diagnosis of rhabdomyosarcoma and the high mitotic rate and arrangement of P.T.A.-positive material in the cytoplasm, as bunches of thin threads, did not fit the diagnosis of oxyphilic granular cell adenoma (onkocytoma). The final diagnosis lay between the latter and leiomyosarcoma. ×370.



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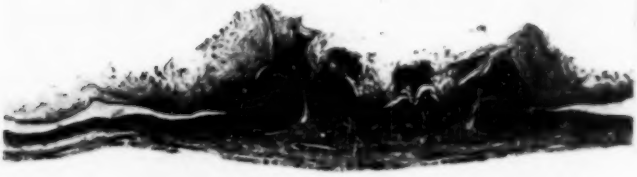

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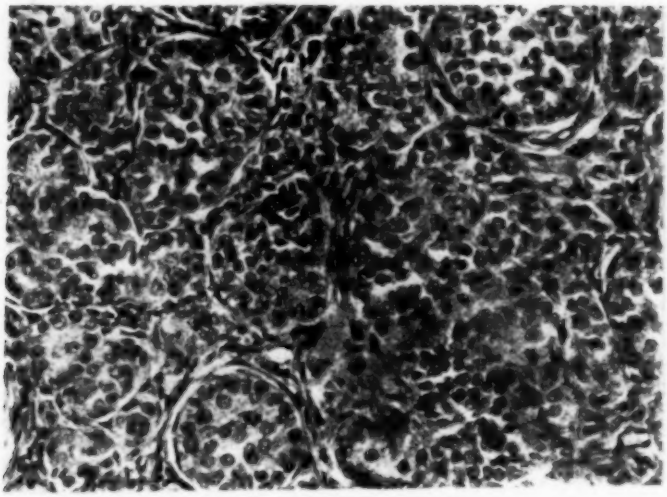

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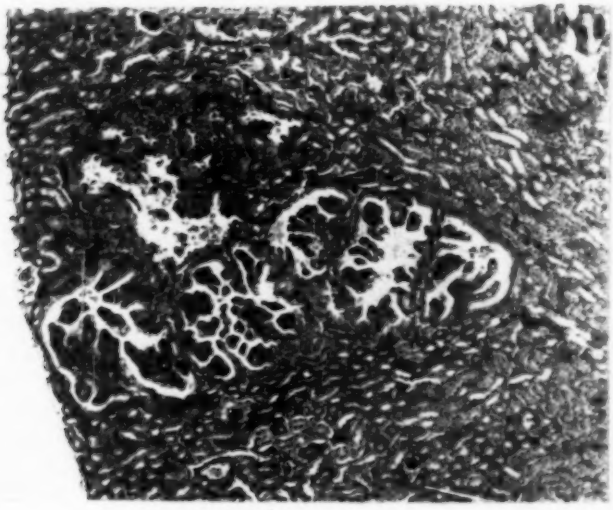

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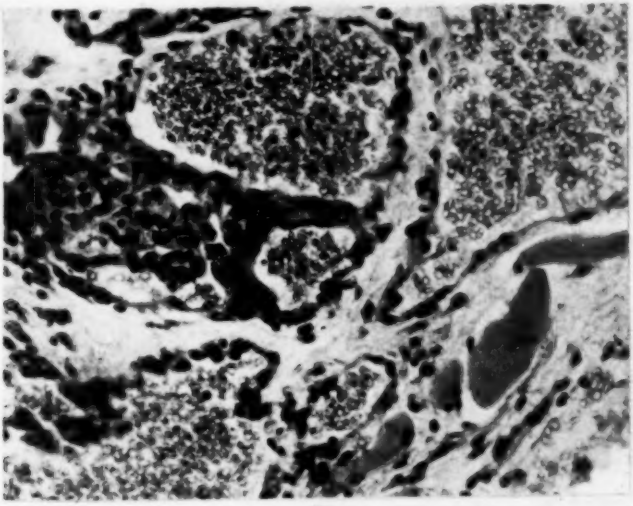

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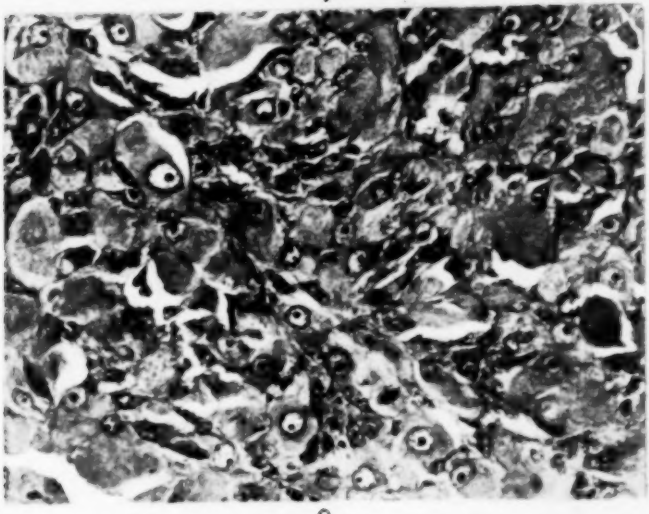

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TABLE V.—*Miscellaneous Tumours seen in 52-week old CBA and "101"-strain Mice, Injected when New-born with 30 μg. DMBA (Excluding Malignant Lymphoma and Tumours of Lung, Skin, Stomach, and Liver)*

Group	Strain	Treatment	Mice examined at 52 weeks	Miscellaneous tumours	Figure Number
1	CBA	DMBA/Gelatine	18	Carcinoma of adrenal cortex (1 case)	5
				Granulosa-cell tumour of ovary (2 cases)	
2	..	Gelatine	25	None	—
3	..	None	39	None	—
4	101	DMBA/Gelatine	25	Cortical adenoma of kidney (2 cases, one with 2 adenomas)	6
				Fibrosarcoma in subcutaneous tissues of ear (1 case)	
				Haemangioma (3 cases, one in subcutaneous tissues of neck, one in uterus, and one highly malignant tumour involving thigh and body wall)	7
				Myeloid leukaemia (1 case)	
				? Leiomyosarcoma of parotid gland (1 case)	8
5	..	None	40	None	—

The occurrence of papillonephritis in "101"-strain mice has been commented upon previously (Roe *et al.*, 1959). The present observation confirms our previous impression that treatment with DMBA does not materially affect the incidence of the disease (Roe and Peirce, 1960).

DISCUSSION

1. *Significance of experimental results*

In general our experimental findings confirmed those of Pietra *et al.* (1959, 1961), Stich (1960), Fiore-Donati *et al.* (1961), and Kelly and O'Gara (1961), but several new observations were made.

Injection of 30 μg. DMBA subcutaneously into new-born mice had no pronounced effect on survival up to the age of weaning, but adversely affected it to a significant degree between the ages of 1 month and 1 year. During the latter period malignant lymphoma (stem-cell type) and multiple lung tumours accounted for about half the deaths, but there was also an increased death-rate from causes other than neoplasia. Cases of malignant lymphoma were seen in the DMBA-treated groups of both strains of mice: all cases arose between the 10th and 32nd weeks, a finding closely similar to that in the earlier studies referred to above. The incidence was similar in the two strains (20 per cent in "101"s and 15·4 per cent in CBAs), but the time of appearance was significantly earlier in the "101" strain. There was no evidence that susceptibility differed between litters of the same strain.

Multiple pulmonary tumours were seen in DMBA-treated mice of both strains. Most of these were apparently benign, but two were manifestly malignant. At one year the average number of pulmonary tumours per survivor was similar in

the two strains, but there was a significant strain difference in the average size of the largest adenoma in each mouse. The fact that all lung tumours (not only the largest) tended to be larger in the " 101 " strain could be explained by earlier appearance in this strain, as in the case of the malignant lymphomas. However, a recent observation by Rogers (1960) throws doubt on this view : he found that equal-sized pieces of small and large lung tumours, when transplanted to the ears of genetically compatible host mice, grew at different rates, the latter growing faster than the former. This would suggest that in the present experiment the lung tumours in the " 101 " strain had a higher growth-rate than those in the CBA strain. Further experiment is required to settle this point.

The results as a whole indicated that the CBA strain is less responsive to the carcinogenic action of DMBA than the " 101 " strain. It was therefore surprising that the final incidence of lung tumours was so similar. Of course the tumour counts refer only to macroscopically visible surface adenomas, and the incidence in the two strains might have appeared quite different if smaller and more deeply situated lesions had been taken into account. Further, the comparison was made after the arbitrary period of one year. If this period had been shorter or longer a difference in tumour incidence might have been seen. The period of onset of malignant lymphomas was complete at the 32nd week. We do not know whether the appearance of lung tumours was confined to a definite period, or continued throughout life ; and, unfortunately, the very recently reported experiments of Kelly and O'Gara (1961) were not carried on long enough to answer this question. In some ways the data on size and incidence of tumours presented here suggests that the former is true. This is a fascinating problem which does not seem to have been adequately studied (see review on pulmonary tumours in experimental animals by Shimkin, 1955).

In our experiments there was no definite correlation between treatment with DMBA and incidence of hepatomas, although it was curious that in DMBA-treated mice they were usually multiple, while in controls two was the most seen in any one mouse. In the experiments of Pietra and his colleagues occasional hepatomas were seen in carcinogen-treated mice, but none in controls. More recently, in a preliminary report, Liebelt, Yoshida, and Gray (1961) describe a striking enhancement of the incidence of hepatomata in C_3Hf mice injected with urethane when less than 24 hours old but not when 6–8 weeks old. The presence of cytoplasmic inclusions, similar to those described by Burns and Schenken (1940) and Craigie (1955), in the hepatomas of both DMBA-treated and control mice in the present experiment suggests that DMBA was not the only causative agent. It may be that the administration of carcinogen to new-born mice enhances the effect of another agent.

The induction of skin tumours by administration of DMBA to suckling mice was perhaps not surprising, in view of certain recent observations. Rous (1956) observed skin tumours in untreated mice, and attributed them to the fact that the mice had been bred in wooden cages preserved with creosote. Poel and Kammer (1957), Lijinsky, Saffiotti, and Shubik (1957), Shubik, Spencer, and Della Porta (1957) and Boutwell and Bosch (1958) conducted experiments which confirmed that relatively slight exposure of infant or adult mice to creosote led to the induction or initiation of skin tumours. The fact that most of the tumours seen in the present experiment arose on the face and head fits in with a previous observation of one of us (Roe, 1956) that the skin tumours which arise 36 weeks or more

after the application of 300 μg. DMBA to the dorsal skin of 8-week old mice are also predominantly on the head and face.

Papillomas of the forestomach were not noted by Pietra and his colleagues. However such tumours would be overlooked if routine post mortem examination did not include dilatation of the stomach with fixative and examination of the lining when fixed. The discovery of papillomas in this site was not altogether surprising, in view of the recent demonstration by Bock and King (1959) of the relatively high sensitivity of the forestomach epithelium to tumour induction.

The telangiectasia of the stomach wall is also a new observation. Dilatation of vessels and haemorrhages in the wall of the small gut, particularly in the proximity of Peyer's patches, in mice treated as adults with DMBA or urethane have been seen by us in previous experiments, but we do not recall seeing similar lesions in the forestomach. Unfortunately no record of the frequency of intestinal telangiectases and haemorrhages was made in the present experiment, though it is known that they occurred. There was no tendency for papillomas and telangiectases of the forestomach to occur at the same site.

Several other miscellaneous tumours, in particular granulosa-cell tumours of the ovary, and haemangiomata of the various sites, occurred both in the present experiment and in those of Pietra *et al.* (1961). There can be little doubt that they resulted from carcinogen treatment. The induction of a carcinoma of the adrenal cortex, of an unusual tumour of the parotid gland (? leiomyosarcoma) (Fig. 8), and of several adenomata of the renal cortex, by a chemical carcinogen are observations which may have importance in their own right.

Search through the literature listed by Hartwell (1951) and Shubik and Hartwell (1957) revealed no examples of induction of any of these tumours by carcinogenic polycyclic hydrocarbons, except the induction of renal tumours by the introduction of massive doses directly into the kidney (Ilfield, 1936 ; Oberling, Sannie and Guerin, 1937 ; Esmarch, 1942). Even this drastic technique sometimes yielded negative results (e.g. Oberling *et al.*, 1936 ; Woglom, 1938). On the other hand, adenomata of the renal cortex have been repeatedly induced by other agents, e.g. by polyoma virus in mice (Stanton *et al.*, 1959) and hamsters (Ham *et al.*, 1960), by another virus in frogs (Lucké, 1952), by oestrogens in male hamsters (Kirkman and Bacon, 1949 ; Horning, 1954), and by X-irradiation in rats (Koletsky and Gustafson, 1955).

Steiner (1942) introduced carcinogenic polycyclic hydrocarbons into the salivary glands of mice, rats, guinea-pigs, and rabbits. In all species except the rabbit he observed a variety of tumours including squamous carcinomas, acantho-adenocarcinomas, adenocarcinomas, spindle-cell sarcomas, and cavernous haemangiosarcomas. However he did not see any tumours similar in microscopic appearance to the one in the present experiment (Fig. 8), and no such tumours are recorded in experiments prior to 1942 (for reference see Steiner, 1942). It is possible that some of the parotid gland tumours seen by Bauer and Byrne (1950) were in fact of this histological type. Like Steiner they induced their tumours by the introduction of the carcinogen (20-methylcholanthrene) directly into the salivary gland.

The parotid tumours induced by the polyoma virus (Law, Dunn and Boyle, 1955 ; Salaman, 1959) are histologically quite different from the tumour seen in the present experiment.

The absence of tumours at the site of injection of DMBA is somewhat sur-

prising, and suggests rapid diffusion, which may prove one of the advantages of the technique when the method is considered as a possible general test for carcinogenicity (see next section).

2. *The possibility of the development of a sensitive, generally applicable, broad-spectrum test for carcinogenic action*

The armoury of tests available for detecting carcinogenic activity is potentially large, but most such tests involve long periods of treatment and/or observation before a positive result can be expected or a negative one accepted. A quick and sensitive screening test for carcinogenic activity is being sought today by many with almost alchemistic fervour. Several have been proposed (e.g. the sebaceous gland suppression test of Suntzeff, Cowdry and Croninger, 1955, and the newt test of Neukomm, 1956) : however, after brief periods of popularity, such short-cut methods have been found inadequate as substitutes for a battery of long-term tests.

The extraordinary sensitivity of new-born mice to the carcinogenic action of a single 30 μg. dose of DMBA given subcutaneously, as first demonstrated by Pietra *et al.* (1959), suggested to us the possibility that this technique might lead to the development of a relatively quick and sensitive test for carcinogenic action. The subsequent studies of Pietra *et al.* (1961), Stich (1960), Fiore-Donati *et al.* (1961), Kelly and O'Gara (1961), and those reported here, are encouraging.

The relevant facts are these :—

(1) All strains of mice so far tested have developed multiple tumours in response to treatment with a known carcinogen, whilst very few tumours have been seen in comparable controls treated with solvent only. Immediate mortality was low.

(2) 4 carcinogenic polycyclic hydrocarbons gave clear-cut positive results, and so did urethane, a carcinogen of an entirely different class.

(3) The induction of tumours of the forestomach, skin, kidney, ovary, etc., clearly indicate that the test material spreads all over the body and that the test is not dependent, as most others are, on the sensitivity of one tissue.

(4) In the case of malignant lymphomas there is evidence of an upper age limit after which this type of neoplasm does not appear. For the Swiss mice used by Pietra *et al.* (1961), and for the CBA strain used by us, this upper limit appears to be around the 33rd week, and for the " 101 " strain some 10 weeks earlier. Thus it may be possible to establish an acceptable " negative " after a definite period of observation.

(5) The sensitivity of lung tissue to tumour induction is perhaps the most striking feature of this test. A significant positive would certainly have been scored using a much lower dose of DMBA than 30 μg. Thus there is hope that very weak carcinogenic activity may be detectable. Pietra *et al.* (1961) reported a 37·5 per cent incidence of pulmonary tumours in mice treated with only 40 μg. urethane, a much smaller dose of this substance than any previously shown to be effective.

(6) Apart from the sensitivity of the test, if generally applicable it will prove very economical. Each mouse is under experiment from the day of its birth, and no space has to be set aside for animals too young for use in experiments.

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A test of this kind can be regarded simply as an improvement on that developed by Andervont and Shimkin during the years from 1935 onwards. Appreciating the extreme sensitivity of mouse pulmonary tissue to the carcinogenic effect of a variety of agents, they injected a standard dose (0·25 mg.) of the test substance intravenously into young adult mice, and recorded the percentage of mice which developed lung tumours. Later the multiplicity and size of induced tumours were taken into account. But the difficulty remained that the test had no clear end-point (see Shimkin, 1955, p. 240). The longer the mice were kept the higher the proportion of tumour-bearers and the number of tumours, in both test and control mice. But when newborn mice are injected, it appears that malignant lymphoma induction has an end-point at about 23 to 33 weeks, depending on the strain, and it is possible that there is an end-point to lung-tumour induction also (*vide supra*, p. 524). This method enables observations to be made earlier, and if there is an end-point it is brought forward to a time when it is easier to detect. Since Andervont and Shimkin made their fundamental studies many changes have taken place in animal husbandry. The importance of contamination of mice during the neonatal period with small amounts of carcinogens has been underlined by recent studies on the use of creosote-treated wooden mouse boxes (Boutwell and Bosch, 1958; Roe, Bosch and Boutwell, 1958). Since the use of metal cages for all breeding and experimental mice became general, the incidence of so-called " spontaneous " lung tumours in mice has declined. There is therefore a real possibility that an end-point to the period during which these tumours appear after exposure to a single early carcinogenic stimulus could now be demonstrated.

The next stages in the development of the test include the assessment of carcinogens of different chemical types and of weak or borderline carcinogens, dose-response studies, and the search for even more sensitive strains of mice, or other species. Clearly many workers in this general field are thinking along these lines. Already data on dose-response relationships are becoming available (e.g. Kelly and O'Gara, 1961), and other experiments are in progress in several research centres.

3. *The bearing of these results on immunological theories of carcinogenesis*

According to a theory which has been put forward in various forms, an immune reaction is one component of the carcinogenic process (e.g. Green, 1961). A chemical carcinogen is supposed to form a complex with a specific cell protein. This complex, it is assumed, is antigenic, and the antibody formed to it acts on both the altered and unaltered protein, creating a state of stress in the treated tissue. Cells then appear by mutation which are deficient in the specific protein, and these, it is thought, will have a proliferative advantage over their neighbours.

This theory, to which the foregoing brief account does less than justice, requires that the antibody-forming apparatus shall be competent while stimulating antigen is present. It has been shown that when an antigen is introduced into a foetal or new-born mouse, and persists in the body, no antibody is formed to it, and a state of specific tolerance develops (for review see Medawar, 1960).

If, as seems probable, the carcinogen-protein complex is formed quickly, the injection of a carcinogen into a new-born mouse ought to give rise not to antibody formation, but to immune-tolerance. Rapid multiple tumour induction by this

method suggests therefore that carcinogenesis is not necessarily dependent on an immune mechanism.

The position is complicated by the facts that only the carcinogen-protein complex is assumed to be antibody-inducing, but that the unaltered protein reacts with the antibody formed in response to the complex. To produce lasting tolerance it has been shown that the antigen must persist (Smith and Bridges, 1958; Mitchison, 1959). It is true that the carcinogen, as well as its protein complex, is probably eliminated fairly rapidly (in a few days to a few weeks, see Heidelberger and Wiess, 1951) but it is impossible to say whether the continued presence of the antibody-reactive original protein would be adequate to maintain the tolerance originally induced by the carcinogen-protein complex. But even if it were not, since it is a " self " protein, it would not itself stimulate antibody production once the age of immune non-competence was passed.

It does not seem possible to avoid the conclusion that carcinogenesis by injection of the new-born is incompatible with this immunological theory of carcinogenesis.

SUMMARY

1. Mice of two strains, CBA and " 101 ", were injected when new-born with 30 μg. 9,10-dimethyl-1,2-benzanthracene (DMBA) in 1 per cent aqueous gelatine, and thereafter examined regularly for tumour development. At the end of one year all the survivors were killed and examined post mortem. Comparable untreated mice of both strains, and CBA mice injected with aqueous gelatine, were observed.

2. DMBA had no definite effect on survival before weaning, but adversely affected it between 1 month and 1 year. Malignant lymphoma (stem-cell type) and tumours of the lung were commonest causes of death. There were no deaths from tumours among the control groups.

3. In the DMBA-treated mice killed after 1 year, tumours of the following sites were seen : lung (average of over 12 tumours per mouse), skin, forestomach, liver, ovary, adrenal, kidney, subcutaneous tissues, parotid gland, and uterus. Occasional tumours of liver and lung, and one malignant lymphoma of the histiocytic type, were the only tumours seen in control mice.

4. Telangiectases were seen in the wall of the forestomach of 8 DMBA-treated mice and of 1 control mouse.

5. In general the " 101 " strain was more sensitive to the carcinogenic action of DMBA : malignant lymphoma, and tumours of skin, forestomach, and miscellaneous sites were more frequent in this strain. Malignant lymphoma appeared after a significantly shorter latent interval, and the lung tumours seen in mice killed after 1 year were significantly larger.

6. The results are compared with those of previous workers, and the possibility of developing a highly sensitive broad-spectrum test for carcinogenic action based on the injection of test substances into newborn mice is discussed.

7. The implications of these results for certain immunological theories of carcinogenesis are considered.

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REFERENCES

Bauer, W. H. and Byrne, J. J.—(1950) *Cancer Res.*, **10**, 755.
Bock, F. G. and King, D. W.—(1959) *J. nat. Cancer Inst.*, **23**, 833.
Boutwell, R. K. and Bosch, D. K.—(1958) *Cancer Res.*, **18**, 1171.
Burns, E. L. and Schenken, J. R.—(1940) *Amer. J. Cancer*, **39**, 25.
Craigie, J.—(1955) *Rep. imp. Cancer Res. Fd.* **52**, 8.
Esmarch, O.—(1942) *Acta path. microbiol. scand.*, **19**, 79.
Fiore-Donati, L., Chieco-Bianchi, L., de Benedictis, G. and Maiorano, G.—(1961) *Nature, Lond.*, **190**, 278.
Green, H. N.—(1961) *Acta Un. int. Cancr.*, **17**, 215.
Gross, L.—(1957) *Tex. Rep. Biol. Med.*, **15**, 603.
Ham, A. W., McCulloch, E. A., Axelrad, A. A., Siminovitch, L. and Howatson, A. F.—(1960) *J. nat. Cancer Inst.*, **24**, 1113.
Hartwell, J. L.—(1951) "Survey of compounds which have been tested for carcinogenic activity." Washington, D.C. (U.S. Government Printing Office.)
Heidelberger, C. and Weiss, S. M.—(1951) *Cancer Res.*, **11**, 885.
Horning, E. S.—(1954) *Brit. J. Cancer*, **8**, 627.
Ilfield, F. W.—(1936) *Amer. J. Cancer*, **26**, 743.
Kelly, M. G. and O'Gara, R. W.—(1961) *J. nat. Cancer Inst.*, **26**, 651.
Kirkman, H. and Bacon, R. L.—(1949) *Anat. Rec.*, **103**, 475.
Koletsky, S. and Gustafson, G. E.—(1955) *Cancer Res.*, **15**, 100.
Law, L. W., Dunn, T. B. and Boyle, P. J.—(1955) *J. nat. Cancer Inst.*, **16**, 495.
Liebelt, A., Yoshida, R. and Gray, G. F.—(1961) *Proc. Amer. Ass. Cancer Res.*, **3**, 245.
Lijinsky, W., Saffiotti, U. and Shubik, P.—(1957) *J. nat. Cancer Inst.*, **18**, 687.
Lucké, B.—(1952) *Ann. N.Y. Acad. Sci.*, **54**, 1093.
Medawar, P. B.—(1960) Ciba Foundation Symposium on 'Cellular Aspects of Immunity'. London (Churchill), p. 134.
Mitchison, N. A.—(1959) 'Biological Problems of Grafting: a Symposium'. Oxford (Blackwell), p. 239.
Neukomm, S.—(1957) *Oncologia*, **10**, 107.
Oberling, C., Sannie, C., Guerin, M. and Guerin, P.—(1936) *Bull. Ass. franç. Cancer*, **25**, 156.
Idem. Sannie, C. and Guerin, P.—(1937) Fifth Conference de la Leewenhoeck-Vereenig, Amsterdam, p. 57.
Pietra, G., Rappaport, H. and Shubik, P.—(1961) *Cancer*, **14**, 308.
Idem, Spencer, K. and Shubik, P.—(1959) *Nature, Lond.*, **183**, 1689.
Poel, W. E. and Kammer, A. G.—(1957) *J. nat. Cancer Inst.*, **18**, 41.
Roe, F. J. C.—(1956) *Brit. J. Cancer*, **10**, 61.
Idem, Bosch, D. and Boutwell, R. K.—(1958) *Cancer Res.*, **18**, 1176.
Idem and Peirce, W. E. H.—(1960) *J. nat. Cancer Inst.*, **24**, 1389.
Idem. Salaman, M. H., Cohen, J. and Burgan, J. G.—(1959) *Brit. J. Cancer*, **13**, 623.
Rogers, S.—(1960) *Arch. Path.*, **70**, 661.
Rous, P.—(1956) *Proc. Amer. Ass. Cancer Res.*, **2**, 143.
Salaman, M. H.—(1959) *Brit. J. Cancer*, **13**, 76.
Shimkin, M. B.—(1955) 'Pulmonary Tumors in Experimental Animals'. *Advanc. Cancer Res.*, **3**, 223.

SHUBIK, P., SPENCER, K. AND DELLA PORTA, G.—(1957) *J. nat. Cancer Inst.*, **19**, 33.
Idem AND HARTWELL, J. L.—(1957) 'Survey of compounds which have been tested for carcinogenic activity', Supplement 1. Washington, D.C. (U.S. Government Printing Office).
SMITH, R. T. AND BRIDGES, R. A.—(1958) *J. exp. Med.*, **108**, 227.
STANTON, M. F., STEWART, S. E., EDDY, B. E. AND BLACKWELL, R. H.—(1959) *J. nat. Cancer Inst.*, **23**, 1441.
SNELL, G. D., STAATS, J., LYON, M. F., DUNN, L. C., GRUENEBERG, H., HERTWIG, P. AND HESTON, W. E.—(1960) *Cancer Res.*, **20**, 145.
STEINER, P. E.—(1942) *Arch. Path.*, **34**, 613.
STICH, H. F.—(1960) *J. nat. Cancer Inst.*, **25**, 649.
SUNTZEFF, V., COWDRY, E. V. AND CRONINGER, A.—(1955) *Cancer Res.*, **15**, 637.
WOGLOM, W. H.—(1938) *Amer. J. Cancer*, **32**, 447.

ENHANCEMENT OF THE PATHOGENICITY OF MOUSE HEPATITIS VIRUS (MHV1) BY PRIOR INFECTION OF MICE WITH CERTAIN LEUKAEMIA AGENTS

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THE pathogenic action of most mouse leukaemia viruses is difficult to study on account of the long and variable time before the appearance of leukaemia. Useful information on the multiplication and pathogenesis of these viruses might be obtained if a virus could be found which modifies their pathogenicity or, alternatively, which has its own pathogenicity modified. Mouse hepatitis virus (MHV1) seemed a suitable choice for such an investigation : its pathogenic action is stable and predictable ; it produces mild hepatitis in normal weanling mice and fatal hepatitis in weanling mice recently infected with *Eperythrozoon coccoides* (Niven, Gledhill, Dick and Andrews, 1952) ; and, it bears a general resemblance to many leukaemia viruses, e.g. in size, thermolability, host specificity (cf. Gledhill, Dick and Niven, 1955, and Moloney, 1960*b*). Experiments described here were undertaken to study dual infections of mice with MHV1 and Friend or Moloney leukaemia agents (Friend, 1957 ; Moloney, 1960*a*). It was found that, while prior inoculation of MHV1 reduced the pathogenicity of Friend leukaemia agent, fatal hepatitis resulted if the hepatitis virus was inoculated several days after the Friend agent. The latter phenomenon closely resembled the enhancement of MHV1 by *E. coccoides*. The Moloney leukaemia agent also enhanced MHV1 provided the inoculation preceded that of MHV1 by at least 20 days but the enhancement was somewhat less marked. The enhancement may provide a useful method for titration of Moloney agent.

MATERIALS AND METHODS

Mice

Two strains of white mice, designated " Albany " and " Swiss ", were used. Both had been bred for many years at the farm of the Division of Laboratories and Research, New York State Department of Health, Albany. In some experiments 1–2 day old mice were employed ; these are termed " baby " mice. In other experiments newly weaned mice aged 20–28 days (8–12 g.) were used ; these are termed " weanling " mice. Albany mice were used for experiments to show enhancement of MHV1 because they appeared more susceptible to MHV1 than Swiss mice. Swiss mice were employed to demonstrate Moloney leukaemia because limited experience suggested that they develop it sooner than Albany mice.

Inoculations

In all experiments described the route of inoculation was intraperitoneal and the inoculum was 0·2 ml. for weanling mice and 0·03 ml. for unweaned mice.

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The diluent of all tissue suspensions for inoculation or storage was a mixture of equal parts 0·85 per cent saline and 10 per cent horse serum broth, referred to as SS broth.

Preparation of virus suspensions

The Friend and Moloney leukaemia agents were obtained as lyophilised tissue suspensions from Dr. Charlotte Friend and Dr. J. B. Moloney. The reconstituted Friend agent was inoculated into Swiss weanlings and passed as a 1 per cent spleen suspension on the 27th day into similar mice. On the 13th day spleen suspension from these mice was passed into Albany weanlings which were killed after 14 days. The supernatant of a centrifuged 10 per cent suspension of their spleens was distributed in small bottles and stored at −80° as a pool for use in experiments.

The reconstituted Moloney agent was inoculated into and passed in baby Swiss mice. In the third pass using lymph node suspensions, a mouse developed leukaemia in 86 days. The supernatant of (1) the centrifuged spleen suspension (M1/S) and (2) the centrifuged lymph node suspension (M1/L) from this mouse were stored separately at −80°. Reconstituted suspension from another original ampoule was inoculated into baby Swiss mice. Some of these were sacrificed after 12 days and liver-spleen suspension inoculated into similar mice. One of these developed leukaemia in 60 days and the supernatant after 3 cycles of centrifugation of a suspension of lymph nodes and thymus (M2/T) was employed in one experiment. Others of the mice inoculated from the second original ampoule were sacrificed at 27 days and a suspension of spleens and lymph nodes was inoculated into baby Swiss mice. One with signs of leukaemia was killed on the 76th day. A lymph node suspension from this mouse was frozen and slowly thawed three times and then centrifuged. The supernatant (M2/L) was stored at −80° for use in another experiment.

Lyophilised liver-spleen suspension of MHV1 was inoculated into weanling Albany mice. A liver-spleen suspension of these was passed into weanling Albany mice which were killed on the 5th day. The supernatant of a centrifuged 10 per cent liver-spleen suspension from these mice was distributed in bottles and stored at −80°. In the experiments to be described mice were inoculated with a 1 : 10 dilution of the stored suspension. Titration of the stored suspension in Albany mice treated two days previously with *E. coccoides* showed that 0·2 ml. of a 1 per cent suspension contained about 100 LD_{50}. In the absence of *E. coccoides* such an inoculum produced mild non-lethal hepatitis in weanling Albany mice.

Assessment of hepatitis and method of titration of potentiating agents

Weanling mice inoculated with MHV1 in the above dose were killed on the 6th day (unless otherwise stated). The surfaces of the liver were examined both before and after they had been bled by severance of the aorta. The liver lesions were recorded in the following way :—

Appearance of liver	Score
No obvious focal lesions	0
A few focal lesions	1
Many focal lesions	2
Coalescence of focal lesions to give generalised liver abnormality	3
Liver bright yellow background with haemorrhagic areas	4
Mouse dead with liver appearance as in the last group	5

Weanling Albany mice inoculated only with MHV1 scored mostly 1, with 0 and 2 scored quite often. About 1 in 20 scored 3 and none scored 4 or 5 during these experiments. In mice inoculated with an agent able to potentiate MHV1, mice which scored 3, 4 or 5 were regarded as infected with the potentiating agent. Thus, when *E. coccoides* was used to potentiate MHV1 in Albany mice, more than half were dead by the 6th day after the virus inoculation and the remainder scored 3 and 4.

The method adopted for the indirect titration of agents which potentiate MHV1 was intraperitoneal inoculation of decimal dilutions of the agent in SS broth into groups of mice in the usual doses. This was followed by the intraperitoneal inoculation of MHV1 in the usual dose. The age of mice used for inoculation of the potentiating agent was selected so that the potentiation would be maximal when they reached 20–28 days, the age at which MHV1 was inoculated. Weanling mice satisfied this condition for Friend agent and baby mice for Moloney agent. A group inoculated with MHV1 without any potentiating agent was also included. As indicated above, any mice which scored more than 2 were regarded as infected with the potentiating agent and the ID_{50} was calculated from the proportion infected at each dilution by the method of Reed and Muench (1938).

For direct titrations, groups of mice were inoculated with dilutions of leukaemia agent, as with indirect titrations. With Friend agent they were killed after 14 days and infected mice then showed obvious splenomegaly. For precision each spleen heavier than 0·2 g. was considered infected. This weight was rather more than double that of the average normal spleen which was 0·09 g. In fact, most infected spleens were much heavier than 0·2 g (mean about 0·9 g.). With Moloney agent, mice were examined daily. Any which developed easily palpable lymph nodes were killed and the diagnosis based on the gross lesions. To terminate an experiment all survivors were killed and diagnosis of leukaemia was again based on lesions seen at autopsy. The ID_{50} was calculated, as in the indirect titration, from the proportion of infected mice in each dilution.

RESULTS

Experiments with Friend agent and MHV1

In a preliminary experiment, MHV1 was inoculated into groups of 10 weanling Swiss mice 7, 4 and 1 days before and 2 and 6 days after inoculation of 1 per cent spleen suspension containing Friend agent. The mice were killed 14 days after inoculation of the Friend agent and spleen of each was weighed. The experiment was repeated with weanling mice of the Albany strain and the results of both experiments are shown in Table I. The most remarkable and unexpected observation was the high mortality from hepatitis in the Albany mice inoculated with MHV1 six days after Friend agent. Although only one Swiss mouse of the corresponding group died, the degree of hepatitis manifested at autopsy was greater than that produced by MHV1 in normal Swiss mice. As will be seen, the mean spleen weights of both strains of mice inoculated with MHV1 7 and 4 days before and 6 days after Friend agent are below those of the groups which received Friend agent only and, except for the Albany group which received MHV1 7 days before Friend agent, the differences are in fact significant at the 0·02 level of probability. In contrast, the mean spleen weights of mice inoculated with MHV1 one day before and 2 days after Friend agent are not below those of the groups

TABLE I.—*The Effect of Dual Infection with MHV1 and Friend Agent in Swiss and Albany Weanlings*

Time of inoculation MHV1 in relation to Friend inoculation (days)	Swiss mice: Mortality from acute hepatitis Mice which died / Mice in group	Swiss mice: Average spleen weight of survivors (g.)	Albany mice: Mortality from acute hepatitis Mice which died / Mice in group	Albany mice: Average spleen weight of survivors (g.)
−7	0/10	0·47	0/10	0·73
−4	0/10	0·57	0/10	0·59
−1	1/10	1·07	0/10	1·25
+2	0/10	1·15	1/10	0·83
+6	1/10	0·42	7/10	(0·27)*
Friend agent only	0/10	0·92	0/19	0·97
Uninoculated	..	..	0/10	0·12

Mice killed on the 14th day after inoculation of 1 per cent suspension of the Friend agent and spleens weighed individually.

* Mean based upon only three observations.

given only Friend agent. In another experiment in which groups of 10 Albany mice were inoculated with MHV1 4, 5, 6, 7 and 8 days after the Friend agent, the mortalities from acute hepatitis were 5, 5, 10, 10 and 10. The mean spleen weights of the 5 survivors in the groups which received MHV1 4 and 5 days after Friend agent (0·74 and 0·88 g.) were not less than that for the group which received only Friend agent (0·74 g.). Other experiments have confirmed that when MHV1 is given between 7 and 4 days before Friend agent, the splenic enlargement due to the latter is reduced ; but the splenic enlargement is not lessened by MHV1 given in the period from one day before to 5 days after the Friend agent. When MHV1 is given six days after the Friend agent splenic enlargement is apparently reduced but the evidence for this is based only on the results depicted in Table I.

The experiments cited, together with others, showed that when the inoculation into weanling Albany mice of Friend agent precedes that of MHV1 by several days the pathogenicity of MHV1 is much increased. In the following experiment this enhancement of pathogenicity was used as an indirect means of titration of the Friend agent and the result compared with the direct method. The dilutions of Friend agent were inoculated into groups of 20 mice and each group was split into equal parts to give two sets of titrations with an uninoculated control group for each set. Eight days later all mice of one set were inoculated with the usual dose of MHV1. The hepatitis in those which died and in the survivors killed on the sixth day was scored as described under Methods. The mice of the duplicate set were also killed 14 days after inoculation of Friend agent and, as indicated earlier, each mouse whose spleen was heavier than 0·2 g. was considered to be positive. The results are shown in Table II. The ID_{50} by the indirect method was $10^{-4.2}$ g. of spleen and by the direct method $10^{-5.5}$ g. of spleen.

Experiments with Moloney agent and MHV1

Preliminary experiments with Moloney agent showed that it enhanced the virulence of MHV1 provided it was inoculated into baby mice 20–28 days before the MHV1 inoculation. In one experiment a group of 10 baby mice was inoculated with suspension of infected Moloney lymph node (M1/L). One died during lactation. The rest were weaned at 21 days old and given the usual inoculation of

TABLE II.—*Direct and Indirect Titration of Friend Agent*

Dilution of Friend agent ($-\log_{10}$)	Indirect titration: Hepatitis scored No. mice graded as 0	1	2	3	4	5	Indirect titration: Mortality Mice which died / Mice in group	Indirect titration: Infection rate Mice infected / Mice in group	Direct titration Infection rate Mice infected / Mice in group
∞	6	4	0	0	0	0	0/10	0/10	0/7
9	5	5	0	0	0	0	0/10	0/10	0/10
8	6	1	3	0	0	0	0/10	0/10	0/10
7	3	4	2	1	0	0	0/10	1/10	0/10
6	4	4	2	0	0	0	0/10	0/10	0/10
5	4	5	0	1	0	0	0/10	1/10	6/10
4	2	3	3	1	1	0	0/10	2/10	6/10
3	0	1	2	1	4	2	2/10	7/10	10/10
2	0	0	0	1	4	5	5/10	10/10	9/9
1	0	0	0	0	1	9	9/10	10/10	10/10
ID_{50}								$10^{-4.2}$ g.	$10^{-5.5}$ g.

Indirect titration

Usual dose of MHV1 inoculated 8 days after Friend agent.

Surviving mice sacrificed on 14th day after Friend inoculation and considered infected with Friend agent when hepatitis score > 2.

Direct titration

Killed on 14th day after Friend inoculation and considered infected with Friend agent when spleen weight $> 0{\cdot}2$ g.

MHV1. Six died of acute hepatitis on the 6th and 7th days and the livers of the remainder killed on the 7th day scored 3, 2 and 1. In another experiment, 8 baby mice were inoculated with Moloney suspension M1/S followed by MHV1 28 days later. When killed, all showed severe hepatitis and scored 3 or more.

An attempt was made to titrate Moloney agent by the potentiation of MHV1. Groups containing 10 or 11 baby mice were inoculated with dilutions of the Moloney lymph node preparation M2/L and a group of similar mice received an inoculation of SS broth only. Twenty-six days later, all mice were inoculated with the usual dose of MHV1 and after a further six days the degree of hepatitis scored in the usual way. The results, shown in Table III, led to a value of $10^{-6.4}$ g. of

TABLE III.—*Indirect Titration of Moloney Agent M2/L*

Dilution of Moloney agent M2/L ($-\log_{10}$)	Hepatitis scored No. mice graded as 0	1	2	3	4	5	Infection rate Mice infected / Mice in group
∞	1	9	1	0	0	0	0/11
6	0	9	1	0	0	0	0/10
5	3	4	0	2	0	1	3/10
4	0	3	2	4	2	0	6/11
3	5	2	3	1	0	0	1/11
2	0	5	0	4	1	0	5/10
1	0	1	1	9	0	0	9/11
ID_{50}							$10^{-6.5}$ g.

Usual dose of MHV1 inoculated 26 days after dilutions of Moloney agent.

Mice killed on 6th day after MHV1 inoculation and considered infected with Moloney agent when hepatitis score > 2.

tissue for the ID_{50}. In another indirect titration, groups of baby mice were inoculated with dilutions of Moloney suspension (M2/T). Twenty days later they were inoculated with MHV1 and the degree of hepatitis scored on the 6th day. The results, very similar to those of the last experiment, are shown briefly in Table IV

TABLE IV.—*Direct and Indirect Titration of Moloney Agent*

Dilution of Moloney agent ($-\log_{10}$)	Indirect titration	Direct titration		
	Mice infected Mice in group*	Mice infected Mice in group*	Time of recognition of leukaemia (days from inoculation)	Mean (days)
∞	0/7	—	—	—
7	0/9	0/9	—	—
6	1/9	1/7	157	(157)†
5	2/10	2/3	96, 157	(127)†
4	3/10	2/7	122, 146	(134)†
3	7/8	5/9	89, 122, 137, 143, 153	129
2	4/10	8/9	104, 122, 122, 122, 150, 153, 157, 157	136
1	7/10	7/8	94, 96, 104, 104, 122, 122, 157	114
ID_{50} g.	$10^{-4.6}$	$10^{-4.9}$		

Indirect titration

Albany baby mice (1–2 days) inoculated with Moloney suspension M2/T.
Usual dose of MHV1 inoculated 20 days after Moloney.
Mice killed on 6th day after MHV1 inoculation and considered infected with Moloney agent when hepatitis score >2.

Direct titration

Swiss baby mice (2–4 days) inoculated with Moloney suspension M2/T.
Mice with signs of leukaemia killed and diagnosis based on gross lesions. Remainder sacrificed 157 days after inoculation and examined for leukaemia.

* At the commencement all groups contained 10 baby mice. The majority of non-specific losses occurred during lactation.
† Means based on only two or less observations.

and led to an ID_{50} of $10^{-4.6}$ g. of tissue. The experiment took 26 days. In order to obtain a direct titration, the dilutions of Moloney agent M2/T used in this experiment were also inoculated into groups of baby Swiss mice. As indicated in Methods, diagnosis of leukaemia was based upon lesions in mice killed during the currency of the experiment and in survivors sacrificed at its termination on the 157th day. The results, shown in detail in Table IV, led to an ID_{50} of $10^{-4.9}$ g. of tissue.

DISCUSSION

The enhancement of the pathogenicity of MHV1 by the infectious agent *E. coccoides* suggests that the enhancement produced by the tissue preparations containing Friend or Moloney leukaemia agents may be due to infectious agents. This view is supported by the fact that even dilute preparations containing Friend and Moloney agents enhance MHV1 and by the observation that Moloney agent must be inoculated as long as 20 or more days before MHV1 to increase its pathogenicity. Furthermore, the fact that for enhancement of MHV1 the times of pre-inoculation of preparations containing Friend agent and Moloney agent are considerably different suggests that different infectious agents are operative in each case and this indirectly supports the contention that Friend agent and

Moloney agent themselves are responsible for the enhancement. Nevertheless, the possibility that the enhancement is due to other factors present in the preparations cannot be excluded until purified preparations of Friend and Moloney agents are available.

Eperythrozöon coccoides enhances the pathogenicity of MHV1 during the period of high concentration of the parasite in mouse tissues (Gledhill, 1956). If this is also true for the enhancement of MHV1 by Friend and Moloney agents, it would imply that Friend agent attains a high titre in 4–7 days and Moloney agent in about 3 weeks. The point of interest, especially in the case of the Moloney agent, is that the virus would be present in high titre long before signs of leukaemia appear. For example, it might be that a certain cell-virus relationship of infrequent occurrence is required to produce the initial leukaemic cell. Indeed, if such were the case, most well adapted viruses which multiply over long periods of time with little or no direct pathogenic action might be potential agents of tumour production.

The capacity of Moloney agent to potentiate MHV1 is less marked than that of Friend agent or *E. coccoides*. Nevertheless it is sufficient to obtain a titration within a month and, since the time to produce leukaemia is so long and indefinite, this appears of considerable practical value. It would be interesting to determine whether other mouse tumour viruses potentiate MHV1.

The smaller amount of splenic enlargement when MHV1 is inoculated about 3–7 days before Friend agent could perhaps be ascribed to some form of viral interference. The smaller amount of splenic enlargement which probably occurs when MHV1 is inoculated 6 days after Friend agent is more difficult to explain: it might be due to the capacity of MHV1 to parasitise and inhibit the division of tumour cells. Experiments to determine whether MHV1 interferes with Moloney agent in the same way as with Friend agent have not been undertaken. Unless the interference were much stronger than that of MHV1 towards Friend agent, it would be very difficult to detect since the onset of leukaemia occurs at times scattered over several months.

SUMMARY

When Friend leukaemia agent was inoculated intraperitoneally into weanling mice from 4 to at least 8 days before intraperitoneal inoculation of mouse hepatitis virus (MHV1), the pathogenicity of the latter virus was much increased.

A similar but somewhat less marked enhancement of the pathogenicity of MHV1 occurred when 1–2 day old mice were inoculated with Moloney leukaemia agent 20–28 days before they were inoculated with MHV1.

The magnitude of potentiation of MHV1 with both Friend and Moloney agents depended on the strain of mice employed. In both cases it was greater in Albany than in Swiss mice.

The potentiation of MHV1 by these two agents can provide an indirect method of titration. With a suspension of Friend agent an ID_{50} of $10^{-4.2}$ g. of spleen was obtained compared with $10^{-5.5}$ g. by a direct method based upon splenic enlargement. Two different suspensions of Moloney agent yielded ID_{50}s of $10^{-6.4}$ and $10^{-4.6}$ g. of tissue by the indirect method. The latter suspension titrated by the direct method gave an ID_{50} of $10^{-4.9}$ g. and took 157 days compared with 26 days for the indirect titration.

MHV1 given 4–7 days *before* Friend agent reduced the amount of splenic enlargement which Friend agent produced in 14 days. MHV1 given 6 days *after* Friend agent also reduced the spleen weight but MHV1 given in the period from about 2 days before to 5 days after the Friend inoculation did not reduce the amount of splenic enlargement.

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REFERENCES

FRIEND, CHARLOTTE.—(1957) *J. exp. Med.*, **105**, 307.
GLEDHILL, A. W.—(1956) *J. gen. Microbiol.*, **15**, 292.
Idem, DICK, G. W. A. AND NIVEN, JANET S. F.—(1955) *J. Path. Bact.*, **69**, 299.
MOLONEY, J. B.—(1960*a*) *J. nat. Cancer Inst.*, **24**, 933.—(1960*b*) Symposium : Phenomena of Tumor Viruses, U.S. Department of Health, Education & Welfare, p. 7.
NIVEN, JANET S. F., GLEDHILL, A. W., DICK, G. W. A. AND ANDREWES, C. H.—(1952) *Lancet*, ii, 1061.
REED, L. J. AND MUENCH, H.—(1938) *Amer. J. Hyg.*, **27**, 493.

THE ACTION OF TUMOURS ON THEIR HOSTS

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An ever-increasing number of biological systems have been shown to be affected in the tumour-bearing host, but there have been few attempts to give a general theory of the processes involved. The situation has perhaps been unnecessarily complicated by the extensive use of animals bearing tumours weighing up to half their body weight, which must make more difficult the recognition of the host systems which are primarily affected. It seems reasonable to suppose that one or two key effects of the tumour on the host begin at an early stage in its growth, and rapidly snowball into the complex of lesions seen later.

As pointed out by Greenstein (1954), there are only two basic ways in which a tumour is able to exert a general effect on its host. The tumour may either abstract from the diet some component which is essential for the well-being of the host, or it may elaborate some toxic agent and discharge it into the host circulation.

Taking the first alternative, the vast literature on the growth requirements of tumours seems so far to have yielded little more than the negative result that tumours require supplies of the protein components, carbohydrates, fats, and accessory food factors, which are required for the growth of normal tissues. There is evidence, however (Babson and Winnick, 1954), that tumours may utilise serum proteins without prior hydrolysis into amino acids. This aspect has been recently reviewed by LePage and Henderson (1960) but it does not seem likely that any such difference between tumour tissue and normal tissue could explain the effects of malignant tumours on their hosts.

One generally accepted property of tumours is their ability to grow and to store nitrogen at the expense of the host. The "nitrogen trap" hypothesis (Mider, Tesluk and Morton, 1948) was based on the suggestion that tumours take up amino acids from, but do not contribute to, the general metabolic pool of the host. Despite more recent evidence that there may in fact be an exchange between tumour and host nitrogen (LePage and Greenlees, 1955; Finlayson, Forsberg and Dreyfus, 1959) it still seems evident that the tumour-bearing host is at a disadvantage compared with the tumour in the competition for dietary nitrogen.

White and Belkin (1945) found that tumours grew at 75 per cent of control rate in mice maintained on a protein-free diet, and similar results have been reported by other investigators. These results show that tumours can obtain their requirements for growth from the body of the host, although obviously not in full measure. There seems little evidence, however, that the ability of tumours to grow at the expense of the host is greater than that of rapidly growing normal tissue. Seegers (1937) showed that pregnant rats placed on a protein-free diet from the 11th day of gestation would produce viable young, although their weight at birth was reduced. Liver will regenerate in starved animals (Williams, 1951; Kennedy and Pearce, 1958), and also in tumour-bearing animals (Paschkis, Cantarow, Stasney and Hobbs, 1955; Straube and Hill, 1956), and a foetus will

grow well in pregnant tumour-bearing rats (Pashkis, Cantarow and Stasney, 1956 ; Paschkis and Cantarow, 1958).

According to a recent review, incorporation studies lead to a similar conclusion, showing that while tumour tissue combines a rapid uptake of amino acids with a marked ability to concentrate them, these properties are shared by foetal tissue, regenerating liver, and the tissues of young growing rats (LePage and Henderson, 1960).

From the evidence so far cited it is difficult to see why the growth of a tumour should have any greater effect on the host than pregnancy. Nevertheless one inescapable fact is that wasting of the host to a greater or lesser extent is one of the consequences of tumour growth. This may range from the failure of tumour-bearing rats to gain weight as rapidly as controls (Begg, Dickinson and White, 1953) to the all-too familiar picture of severe cachexia in the tumour-bearing patient or rodent. Sherman, Morton and Mider (1950) showed that those tissues which relinquish their protein during starvation also yield up their nitrogen to the tumour, with the exception of liver, spleen, and kidney, which retain nitrogen in tumour-bearing but not in starved animals. The rate of glycine incorporation into protein follows a similar pattern, glycine being more readily incorporated into the liver and spleen of tumour-bearing mice and less readily into muscle than in control mice (Norberg and Greenberg, 1951). Thus simple inanition resembles, but does not simulate exactly, the situation in the tumour-bearing host.

There are in fact many reports in the literature that liver weight and nitrogen content are increased in the tumour-bearing animal. It is, however, often difficult to find out whether the liver weight/body weight ratio also increased, and few investigators state clearly whether " body weight " includes the weight of the tumour or not. Yeakel (1948) found that the livers of tumour-bearing rats increased in weight, and Yeakel and Tobias (1951) that the liver nitrogen content was proportional to the total mass of the animal. Bodansky and Scholler (1956) found an increase in the liver weight/total weight ratio in their tumour-bearing rats. Appleman, Skavinsky, and Stein (1950) found an increased liver weight/total weight ratio in tumour-bearing rats, but not in rats kept on a protein-free diet. An increased protein content was observed in the livers of tumour-bearing rats by Allison, Bernstein, and Babson (1954), and a rapid loss of liver protein in animals kept on a protein-free diet. Similar results were obtained by LePage, Potter, Busch, Heidelberger and Hurlbert (1952) in a comparison of fasted rats with tumour bearers. Once again, however, a comparison with the pregnant rat shows a similarity : the livers of such animals increase considerably in size, although approximately in proportion to the total weight (Poo, Lew and Addis, 1939). These authors concluded that there was a 28 per cent increase in the amount of protein allocated to the liver during pregnancy. Their figures also show that the total weight of pregnant rats increased from 150 to 231 g. in 18 days, compared with an increase to 172 g. in non-pregnant controls. Since the foetal weight on the 18th day was 27 g. it would appear that the host carcass gained weight at least as fast as the non-pregnant controls during this period of rapid foetal growth, although the absence of figures giving the weight of the uterine contents makes any precise assessment difficult. In experiments with pregnant mice to check this point, reported in the present paper, an increase in carcass weight was observed. It seems probable that the carcass of the pregnant

animal is capable of maintaining or gaining weight so long as the diet is adequate. If the diet is not adequate for the support of both mother and foetus then foetal growth will continue at the expense of the maternal tissues. In this sense therefore there is a similarity between the tumour-bearing animal and the pregnant animal on an inadequate diet.

Among the most striking and universal effects of malignant tumours in rodents is the associated fall in liver catalase activity. According to Greenstein and Andervont (1943) liver catalase is unaltered during pregnancy, and this reflects yet another difference between the growth of foetal and tumour tissue in their effects on the host. Studies on the catalase-depressing action of tumours have fallen into two main categories. Firstly the effects of starvation or protein deficiency have been examined, usually in an attempt to simulate the catalase changes in the tumour-bearing host. Appleman, Stavinsky and Stein (1950) found that a protein-free diet depressed rat liver catalase activity to about the same extent as did the growth of the Jensen rat sarcoma. The addition of protein to the deficient diet caused a restoration of catalase activity to normal within 1–3 days—a period similar to that found by Greenstein and Andervont (1942) for the return to normal of the catalase activity of tumour-bearing rats following surgical removal of the tumour. However, a high protein diet (Weil-Malherbe and Schade, 1948) or force-feeding (Begg and Dickinson, 1951), did not restore the catalase activity of tumour-bearing animals to normal. This seems further evidence that the tumour-bearing host has been placed in a position of inability to utilise sufficient dietary protein for its needs.

Secondly the effects of injecting tumour homogenates or fractions have been studied. So far as liver catalase is concerned there is now considerable evidence that the injection of these into normal animals results in changes resembling those seen in tumour bearers. However, as recently pointed out (Adams, 1959, 1961), there is some doubt whether the action of such injections in depressing catalase activity has relevance to the lowered catalase activity seen in the tumour-bearing host.

It is quite possible that the tumour agent affects catalase activity indirectly, by interfering with the utilisation of dietary protein by the host. There are two ways in which this could be brought about. Firstly, the tumour agent may alter the hormonal status of the host animal. Adams (1951, 1952) showed that adrenalectomy in mice resulted in a decreased liver catalase activity (although apparently not in rats (Begg, 1958)), and that both the testicular and adrenal hormones which regulate catalase activity were antagonised *in vivo* by an agent present in tumour homogenates. Recent work (Adams, 1959, 1960) has shown that androgenic hormones and tumour homogenate injections altered the intracellular distribution of liver catalase in opposite directions, and suggested that androgens increase the permeability of large granule membranes to catalase while an agent present in the tumour homogenates decreases it.

Considering adrenal secretion for the moment, an action of the tumour agent in rendering some target tissues insensitive to the action of adrenal hormones might thus affect liver catalase and moreover might well produce the adrenal enlargement often noted in tumour-bearing animals. In his recent review Begg (1958) suggested that " the large adrenal of the tumour-bearing animal is hyperfunctional and is producing considerable quantities of adrenal hormones ". Since Long and his co-workers showed that the administration of adrenal cortical

extracts to animals resulted in an increased nitrogen excretion (e.g. Long, 1942), a markedly increased adrenal secretion in the tumour-bearing animals would be expected to increase tissue catabolism in the host. It is true that in the pregnant rat there is also evidence of an increased adrenal cortical secretion, but this is only between the 10th and 15th days of pregnancy and the figures suggest that the highest secretion rate is only about two or three times the normal (Poulton and Reece, 1957). It is significant that these authors found no increase in adrenal weight at any stage of pregnancy. Thus adrenal hyperfunction appears to be very much less in pregnant that in tumour-bearing animals.

Secondly the tumour agents may be a riboflavin antagonist or anti-metabolite. It has been found that the liver of the tumour-bearing animal contains only about two-thirds of its normal riboflavin (Masayama and Yokoyama, 1939 : Robertson and Kahler, 1942). This may be compared with the observation of Vivanco (1935) that the livers of rats which had died of riboflavin deficiency still contained about one-third of the normal riboflavin content. There is evidence of a close association between riboflavin and nitrogen retention : Czackes and Guggenheim (1946) found that on a low protein diet rats were unable to retain or use riboflavin. Rats on a high protein diet required twice the normal quantity of riboflavin. According to Kaunitz, Weisinger, Blodi, Johnson and Slanetz (1954) riboflavin and protein are mutually limiting factors. Diets with a high protein content cannot be utilised if their riboflavin content is restricted. Adams (1955) found that the liver catalase activity of riboflavin deficient mice did not respond to testosterone, and in the light of more recent work (Adams, 1960) this suggests that riboflavin may also be a factor controlling intracellular catalase distribution.

The present experiments were devised to contribute towards the solution of the problem of how tumours produce their observed effects on the host. Body and liver weights, liver total N, liver weight/body weight ratio, total, granule, and EPC liver catalase activities, and intracellular catalase distribution, have been measured in mice bearing Sarcoma 37, pregnant mice, and mice kept on diets deficient in protein and/or riboflavin with or without treatment with cortisone.

MATERIALS AND METHODS

Animals.—Young adult mice of the 101 and CBA strains were used. These animals were bred in this laboratory by brother-sister mating. The animals were given water *ad libitum*.

Diets.—Three basic types of diet have been used.

(1) Commercial rat cubes (normal diet). Mice were fed this diet unless otherwise stated.

(2) A protein and riboflavin-free diet of the composition :

	Per cent
Starch (maize)	80
Sucrose	10·7
Agar	1·0
Arachis oil	5·0
Salt mix	2·0
(Hubbel Mendel and Wakeman, 1937)	
Streptomycin	0·25
Succinyl Sulphathiazole	0·25
Vitamin mix (no riboflavin)	0·8
(Adams, 1955)	

This diet was supplemented as required with riboflavin (5 μg./g.), and with vitamin-free casein. In the latter case the quantity of starch was reduced to maintain a constant composition of the other components.

(3) One of the problems associated with diets using vitamin-free casein as the sole protein source is that there is a tendency for the animals to reduce their overall food intake. This is probably due to the unpalatable nature of the diet. In an effort to overcome this, particularly in those experiments designed to study the effects of riboflavin deficiency associated with a reduced protein intake, the following diet was designed.

Ground rice	80	per cent
Oatmeal	4	" "
Sucrose	7·7	" "
Arachis oil	As in diet (2)	
Salt mix		
Streptomycin		
Succinyl sulphathiazole		
Vitamin mix (no riboflavin)		

This provides a palatable diet containing about 6 per cent of protein and 0·1 μg./g. of riboflavin (low protein diet). Young adult mice placed on this diet increased their body weight slowly when a riboflavin supplement (5 μg./g.) was added, and lost weight slowly in the absence of such a supplement. However, both groups consumed similar amounts of diet.

In certain experiments vitamin-free casein (15 per cent) was added to the diet with a corresponding reduction in the ground rice content. Groups of young adult mice placed on this diet, with or without a riboflavin supplement of 5 μg./g., did not reduce their food intake, and consequently the observed differences between them were due to riboflavin deficiency uncomplicated by a reduction in food intake.

Diets (2) and (3) were moistened with sufficient water to produce a thick dough, which was cut up into small " biscuits " and dried at slightly above room temperature. Riboflavin supplements were normally added by dissolving the appropriate amount of riboflavin in the water before addition to the powder.

Because the purpose of the work was to simulate the tumour-bearing host, the experimental animals were allowed to eat their diets *ad libitum*.

Preparation of liver fractions and estimation of liver catalase activity.—Catalase estimations were made on whole liver homogenates, and on granule and EPC fractions and given in arbitrary units/mg. N of the whole homogenate. The method of estimation of catalase activity has been fully described in previous publications (Adams, 1950, 1952), and the preparation of the homogenates and fractions by Adams and Burgess (1957, 1959*a*). Ethanol (final concentration 0·01 M.) was added to all catalase-containing solutions, to prevent loss of catalase due to " Complex II " formation (Chance, 1950; Adams and Burgess, 1959*b*).

Liver nitrogen.—The figures quoted are for the total quantity of nitrogen per liver, estimated by the Kjeldahl method.

Tumour.—Sarcoma 37 was obtained originally from the Imperial Cancer Research Fund Laboratories, and maintained by serial passage in this laboratory.

Triton X 100.—This non-ionic detergent (kindly given to us by Charles Lennig

& Co.) was used at a final concentration of 0·25 per cent v/v to disrupt large granules and liberate their catalase activity into solution.

Cortisone.—" Cortisyl " (Roussel) (25 mg. cortisone/ml.) was injected intraperitoneally.

Riboflavin.—Where necessary this was injected intraperitoneally in the form of " Beflavit " (Roche).

RESULTS

The tumour-bearing animal

Fig. 1 shows the effect of the growth of Sarcoma 37 on the liver catalase activity and intracellular distribution, and on liver weight, liver total N, and body weight of CBA female mice. By the end of the third week after the implantation

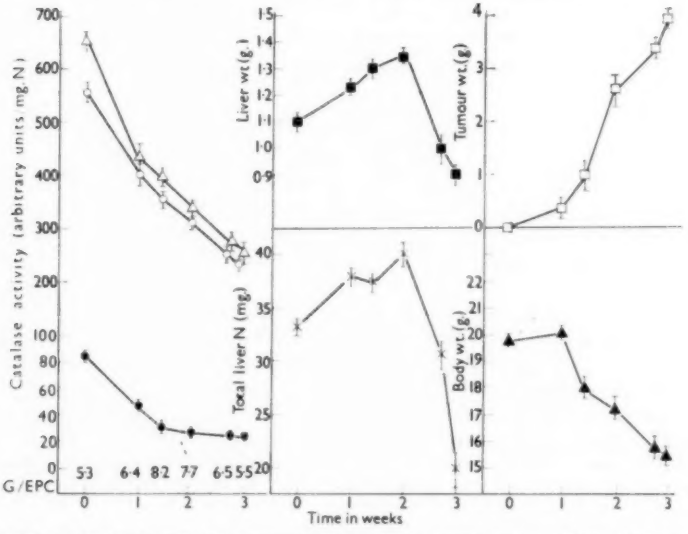

FIG. 1.—Effect of the growth of Sarcoma 37 on CBA female mice. Total liver catalase △———△, granule catalase ○———○, EPC catalase, ●———●, catalase intracellular distribution (G/EPC), liver weight ■———■, total liver nitrogen ×———×, host weight (i.e. without the tumour) ▲———▲. The rate of tumour growth is given by □———□. In this and subsequent figures the results are given as arithmetic ± standard error of means, with 6–8 animals per group. Catalase activities are in arbitrary units/mg. N, liver weight as total net weight, total liver N in mg. per whole liver. Controls are at time 0.

of the tumour the animals were becoming moribund. Total and granule catalase activities decreased progressively throughout the experiment, while the EPC catalase decreased more rapidly than the granule catalase in the first two weeks, and subsequently decreased more slowly. The granule/EPC catalase distribution ratio increased to a maximum value of about 8 as previously reported, but decreased again as the animals became moribund. Liver weight and total N rose for the first two weeks, and then fell sharply. However, the changes in liver N approximately paralleled the changes in wet weight i.e. there was little

or no change in N concentration. Carcass weight fell after the first week and by the terminal stages had decreased by 20–25 per cent. The ratio of the liver weight/total weight increased to about 0·7 until the terminal stages when it returned to normal.

The changes in liver weight/total weight ratio appear in Table I, and the data obtained from all other experiments are also collected in this table.

TABLE I.—*Liver Weight/Body Weight Ratios in Female Mice Subjected to Various Treatments. In the Tumour-bearing and Pregnant Animals "Body Weight" means Total Weight*

Treatment	Time of treatment (weeks)										
	0	0·5	1	1·5	2	2·5	3	4	5	6	7
S37 growth (Fig. 1)	0·53	—	0·59	0·69	0·72	0·54	0·48	—	—	—	—
Pregnancy (Fig. 2)	0·5	—	0·53	—	0·56	0·53	—	—	—	—	—
Protein-free diet + riboflavin (Fig. 3)	0·51	0·49	—	0·5*	0·55	—	—	0·45	—	—	—
Protein-free diet, no riboflavin (Fig. 4)	0·51	0·51	—	0·5*	0·54	—	—	0·54	—	—	—
Rice oatmeal (6%P) diet + riboflavin (Fig. 5)	0·55	—	0·58	—	0·58	—	0·56*	0·54	—	—	—
Rice oatmeal (6%P) diet, no riboflavin (Fig. 6)	0·55	—	0·58	—	0·50	—	0·55*	0·56	—	—	—
Riboflavin deficiency (Fig. 7)	0·51	—	—	—	0·53	—	—	0·51	—	—	0·49
Rice oatmeal (6%P) diet + riboflavin + cortisone (Fig. 8)	0·51	—	—	0·83	—	0·65	—	0·58	—	—	—
Ditto without riboflavin (Fig. 9)	0·51	—	—	0·78	—	0·65	—	0·5	—	—	—

* Protein supplement added to diet after this reading.

The pregnant animal

The effect of pregnancy on the various parameters is shown in Fig. 2. Because of the uncertainty about the date of the beginning of pregnancy the graphs are plotted against foetal weight instead of time. However, since the gestation period of the mouse is about 3 weeks, the abscissae in Fig. 1 and 2 are approximately on the same time scale. In contrast to the tumour bearing animals, granule catalase activity in the pregnant mice did not fall, and even rose slightly. The EPC catalase activity fell significantly towards the end of gestation, but never reached the low level seen in the tumour bearers. However, the granule/EPC catalase distribution ratio rose steadily in the pregnant animals to a value of 9·2, which exceeds the value seen in the tumour bearers. There was an increase in carcass weight, which approximately paralleled that seen in the non-pregnant controls. Liver weight and total N rose considerably, but only in proportion to the total weight of the mouse.

Effect of protein-free diets

The effect of maintaining 101 female mice on a protein- and riboflavin-free diet (diet (2)) is shown in Fig. 3, and on a protein-free diet supplemented with riboflavin in Fig. 4. After 10 days, the catalase activities had fallen, and the

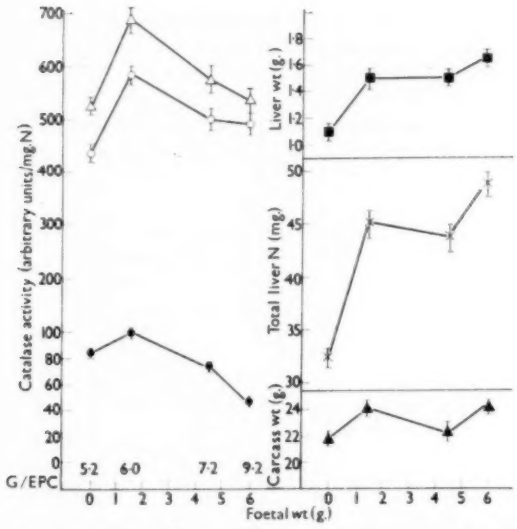

FIG. 2.—Effect of pregnancy on total liver catalase △———△, granule catalase ○———○, EPC catalase ●———●, catalase distribution (G/EPC), liver weight ■———■, total liver N ×———×, carcass weight (i.e. without foetuses, placentas and uteri) ▲———▲. CBA female mice.

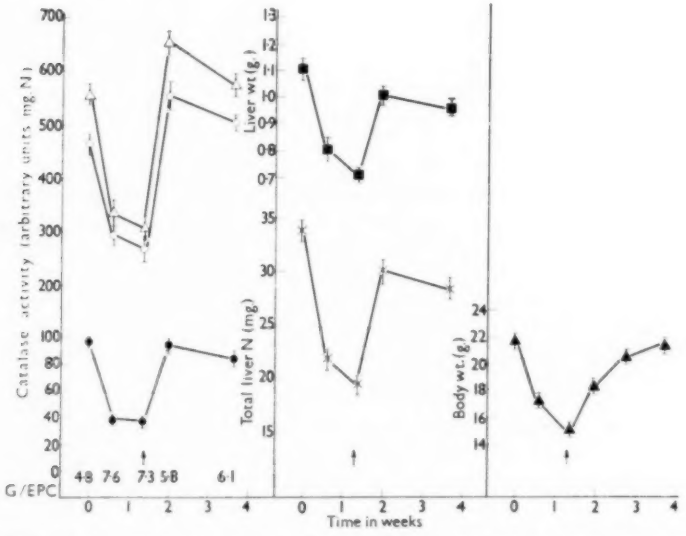

FIG. 3.—Effect of a protein-free diet supplemented with riboflavin on total liver catalase △———△, granule catalase ○———○, EPC catalase ●———●, catalase distribution (G/EPC), liver weight ■———■, total liver N ×———×, and body weight ▲———▲. At the points marked ↑, 15 per cent of vitamin-free casein was added to the diet. 101 female mice.

intracellular distribution had risen, in both groups to about the values found in the tumour bearers. However, in contrast to the tumour bearers, there was no increase in liver weight or total N and the liver weight/body weight ratio remained unchanged. Body weight was lost very rapidly, more rapidly in fact than from the carcass of the tumour bearers. After the 10th day vitamin-free casein (15 per cent final concentration) was added to the diet. In both groups this was followed within 4 days by a return of catalase activity to normal, and an increase in liver weight and total N and in body weight. In the riboflavin deficient group, however, the increase in body weight and liver N was not maintained.

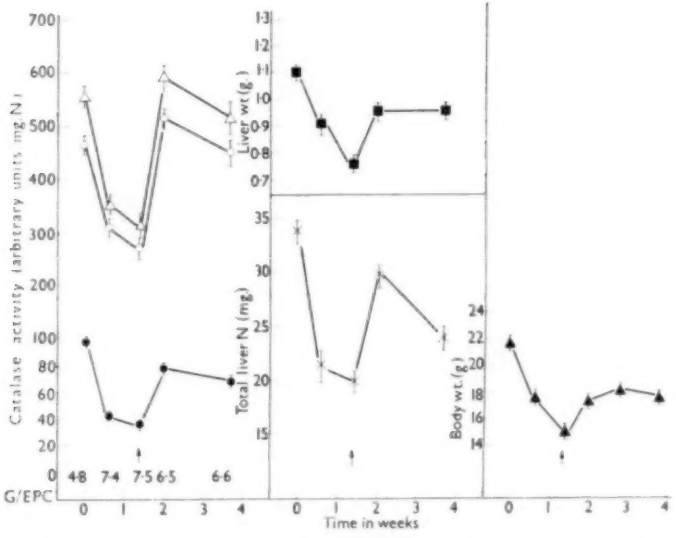

FIG. 4.—As Fig. 3 but without any addition of riboflavin to the diet.

Low protein, low riboflavin diets

Further experiments were carried out using the 6 per cent protein rice-oatmeal diet (diet (3)). As Fig. 5 shows, mice placed on this diet plus a riboflavin supplement maintained or increased their liver and body weights, and there was a slight fall in liver total N. There was little or no change in granule catalase activity, but the EPC catalase activity fell sharply and the average granule/EPC ratio was about 10 from the first to the third week. In the group receiving no riboflavin supplement (Fig. 6) liver weight and total N and body weight all fell slowly. The granule catalase activity decreased, as well as the EPC catalase activity, giving an increase in distribution ratio to about 8. In both groups there was no change in the liver weight/body weight ratio. When the diets of both groups were supplemented with vitamin-free casein (15 per cent) after 3 weeks, catalase activities and distribution ratios rapidly returned towards normal.

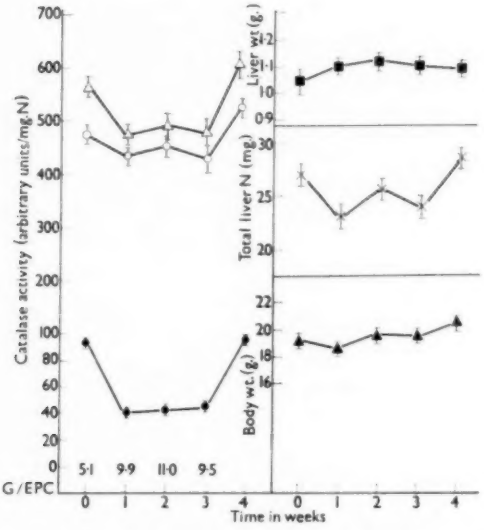

FIG. 5.—Effect of a rice-oatmeal diet containing 6 per cent of protein on total liver catalase △——△, granule catalase ○——○, EPC catalase ●——●, liver weight ■——■, total liver N ×——×, and body weight ▲——▲. CBA female mice.

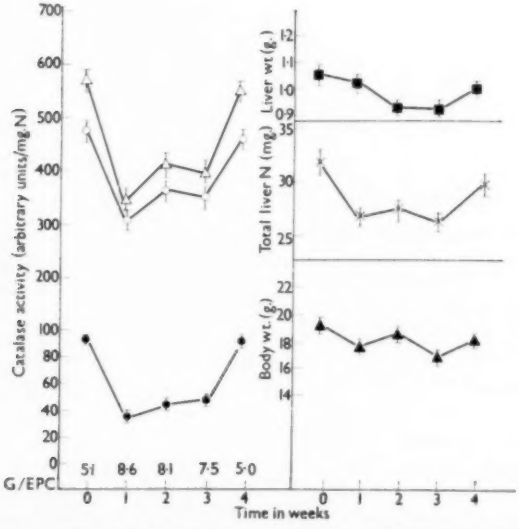

FIG. 6.—As Fig. 5, but without any addition of riboflavin to the diet.

Riboflavin deficiency alone

Fig. 7 shows the effect of riboflavin deficiency alone. In this experiment two groups of mice were fed on diet (3) supplemented with 15 per cent casein, and in the case of one group, with riboflavin. There was no change in the EPC catalase level in either group, but the granule catalase activity fell in the riboflavin-deficient group, with a consequent decrease in the granule/EPC distribution ratio to about 4·4.

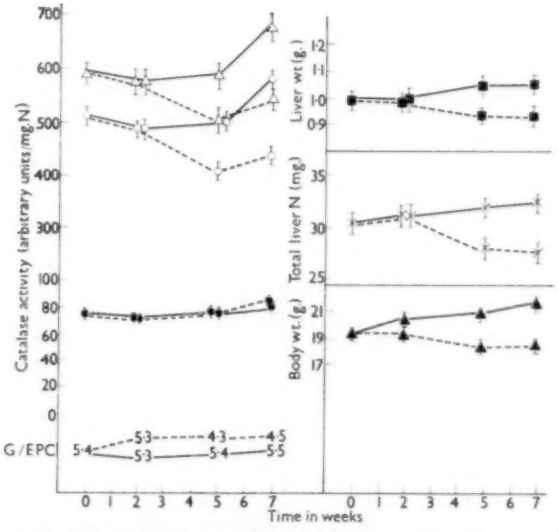

FIG. 7.—Effect of riboflavin deficiency on CBA female mice. The mice were fed on a rice-oatmeal (6 per cent protein) diet supplemented with 15 per cent of vitamin-free casein, supplemented with riboflavin (points joined by continuous lines) and without a riboflavin supplement (points joined by dotted lines). Total catalase △, granule catalase ○, EPC catalase ●, catalase distribution (G/EPC) liver weight ■, total liver N ×, body weight ▲.

Cortisone injections in association with a low protein diet

In further experiments mice were placed on diet (3), half being given a riboflavin supplement, the others not. After 1 week each group was divided into two, and one half of each group injected with 1·5 mg. of cortisone/day. The results appear in Fig. 8 (riboflavin supplemented) and Fig. 9 (riboflavin deficient). They show that the cortisone injections accelerated the fall in total catalase activity, particularly in the riboflavin-deficient group, and restored the granule/EPC distribution ratio to normal in the case of the riboflavin-supplemented group, and to that characteristic of riboflavin deficiency without protein deficiency in the riboflavin-deficient group. Associated with the marked fall in catalase activity was a fall in body weight and an initial rise in liver weight and liver N. The liver weight/body weight ratio was increased to about the value found in tumour bearing animals. After 2–3 weeks of continuous treatment the riboflavin-deficient animals began to die.

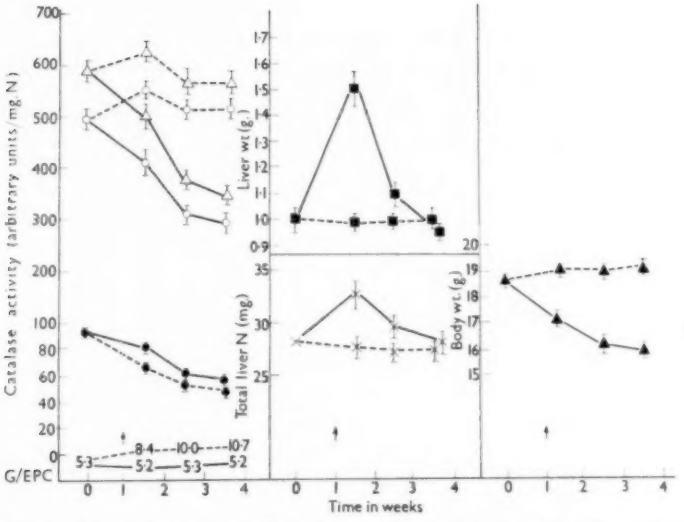

FIG. 8.—Effect of the daily injection of cortisone (1·5 mg./day) on CBA female mice maintained on a rice-oatmeal (6 per cent protein) diet supplemented with riboflavin. Mice were placed on the diet at time 0. After 1 week (↑) they were divided into two groups, one being given cortisone (points joined by continuous lines) and one not (points joined by dotted lines). Total catalase △, granule catalase, ○, EPC catalase ●, liver weight ■, total liver N ×, body weight ▲.

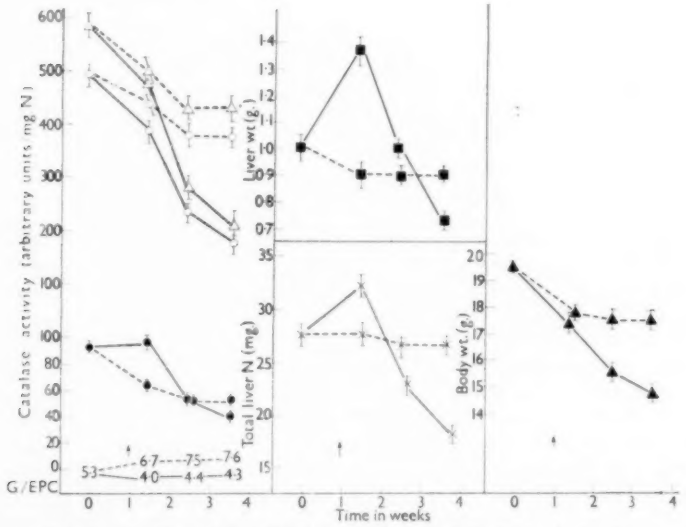

FIG. 9.—As Fig. 8, but without any addition of riboflavin to the diet.

In a separate experiment mice were placed on diet (3) with and without riboflavin for two weeks, and then injected with 1·5 mg. cortisone/day for ten days. The diet of both groups was then supplemented with 15 per cent casein, and the cortisone injections were continued. In the group receiving riboflavin there was a rapid response to the protein supplement, catalase activity and body weight returning approximately to normal after a few days. Of the nine riboflavin-deficient mice remaining at this point, five showed no reaction at all to the protein supplement, and in fact continued to lose weight. The catalase activity and body weight of the other four mice did rise but not to the extent of those receiving riboflavin (Fig. 10).

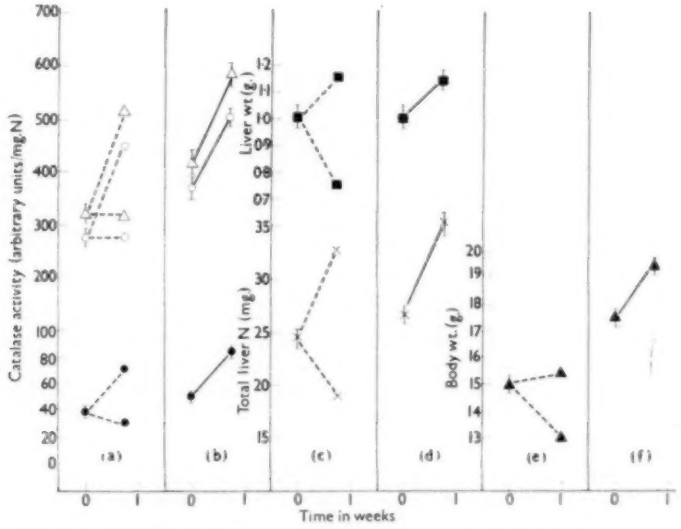

FIG. 10.—Effect of the supplementation with vitamin-free casein (15 per cent final concentration) of mice maintained on a rice-oatmeal (6 per cent protein) diet for 3 weeks, and injected after 1 week with 1·5 mg. cortisone/day. Half the mice received a riboflavin supplement, the other half not. Time 0 represents the situation after 3 weeks on the diet, and the protein supplement was added at this point. Graphs (*a*) (*c*) (*e*) no riboflavin, graphs (*b*) (*d*) (*f*) with riboflavin. In graphs (*a*) (*c*) and (*e*) half the animals responded to the protein supplement and half not (see text). Each group is therefore represented separately.

Pregnant mice treated with cortisone

Table II shows that the raised catalase granule/EPC distribution ratio resulting from pregnancy was restored approximately to normal in mice treated with 1 mg. of cortisone/day for 4 days.

Effect of cortisone injection or riboflavin supplementation in the tumour-bearing animal

A number of male CBA mice were divided into 3 groups. Two groups were maintained on a normal cube diet, and the third group on a diet of cubes moistened with a riboflavin solution and subsequently dried, giving an extra 200 μg./day of

TABLE II.—*Effect of the Injection of 1 mg. Cortisone/day for 4 days on Catalase Activity and Distribution of Pregnant CBA Mice. Catalase Activities are given in Arbitrary Units/mg. N Arithmetic Means ± Standard Error of Means. Six Pregnant Mice per Group and 8 Non-pregnant Controls not Injected with Cortisone*

	Mean foetal weight (g.)	Granule catalase	EPC catalase	G/EPC
Pregnant mice injected with cortisone	4·8	410±11	70±3·2	5·8
Pregnant mice not injected with cortisone	4·5	500±15	74±3·5	7·4
Non-pregnant controls	—	435±7·5	84±2·0	5·2

riboflavin. After one week all these groups were inoculated with Sarcoma 37. The group receiving the riboflavin-supplemented diet was also given a further 200 μg./day of riboflavin by injection. One of the two remaining groups was injected with 1·5 mg. of cortisone daily.

In the cortisone-treated group the rate of S37 growth was reduced, but the animals began to die when the tumour weight reached about one gram. As shown in Fig. 11 and 12 no difference in total catalase activity or granule/EPC distribution ratio was seen comparing the untreated and cortisone treated tumour bearers. It is also evident that supplementing the diet of tumour bearers with riboflavin had little effect.

Dietary intake

The dietary intake of mice kept on the various regimes was measured and the results are summarised in Table III. It seems clear from these results that the various regimes did not produce their effects by causing the animals to restrict their food intake.

TABLE III.—*Dietary Intake of Mice Maintained on the Various Regimes. The Food Intake was Measured Daily (in grams) and Averaged over Weekly Periods*

Regime	Weeks 1	2	3	4	5	6	7
Cubes	4·3	4·0	4·3	5·1	5·2	5·5	—
Protein-free diet + riboflavin	4·0	*5·8	5·2	—	—	—	—
Protein-free diet, no riboflavin	3·3	*5·8	3·7	—	—	—	—
Rice-oatmeal diet (6%P) + riboflavin	4·5	4·6	4·1	*5·0 †4·6	5·2	—	—
Rice-oatmeal diet, no riboflavin	4·9	4·3	4·6	*4·8 †4·8	4·5	—	—
Pregnant, fed cubes	—	5·5	6·0	—	—	—	—
Tumour bearers, fed cubes	4·0	5·2	6·5	—	—	—	—
Riboflavin-deficient	4·0	2·8	3·1	3·0	2·9	3·1	3·2
Controls for above (with riboflavin)	3·2	2·3	3·9	3·1	3·1	3·1	3·3

* Casein added to diet at end of previous week.
† Cortisone (1·5 mg./day) injected at end of previous week.

DISCUSSION

The changes in the various parameters measured, using the various diets, are summarised in Table IV. The results obtained using cortisone are not included in the table.

The results show that pregnancy does affect liver catalase activity in mice, in contrast to the report of Greenstein and Andervont (1943) that catalase activity remained normal in pregnant rats. However, the effects were much less severe

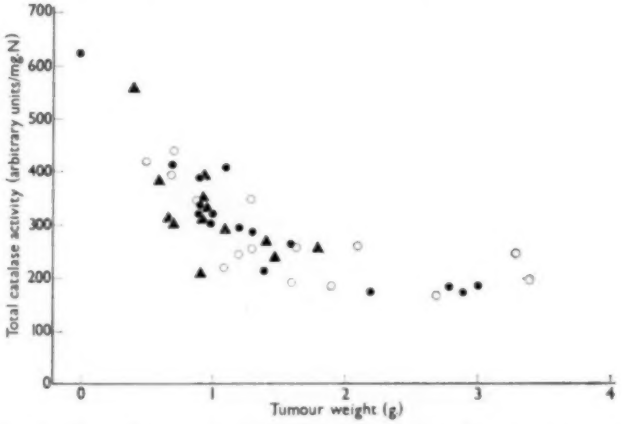

FIG. 11.—Total catalase plotted against tumour weight in S37-bearing CBA male mice.
● Tumour bearers only.
○ Tumour bearers maintained on a high riboflavin diet and injected with riboflavin (see text).
▲ Tumour bearers injected with 1·5 mg. cortisone daily.

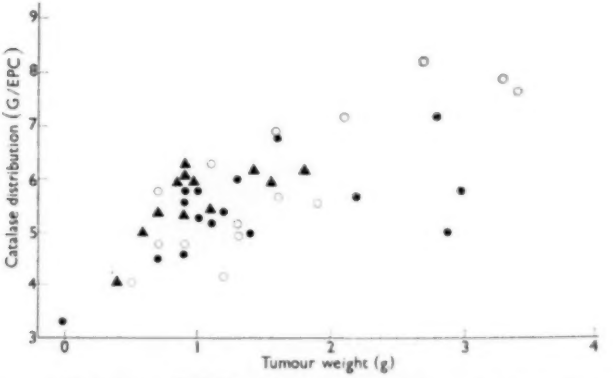

FIG. 12.—Catalase distribution (G/EPC) plotted against tumour weight in Sarcoma 37-bearing CBA male mice. Symbols as in Fig. 11.

than with tumour-bearing animals. The pregnant mice also supported the growth of 6 g. of foetal tissue plus about 2 g. of placental and uterine tissue over a period of 18 days without loss of carcass weight. In contrast the tumour bearers lost 20–25 per cent of their body weight while supporting the growth of about 4 g. of tumour tissue over the same period. The liver weight and total N increased in

Table IV

Animal	Total catalase	Granule catalase	EPC catalase	G/EPC	Liver weight	Liver total N	Carcass weight	Liver weight/body weight
Tumour bearer . . .	Low	Low	Very low	8 (max.)	First increased then decreased	First increased then decreased	20–25% loss in 21 days	Increased
Pregnant	High	High	Low	9·2 (max.)	Increased	Increased	Maintained	Normal
Normal on protein-free diet .	Low	Low	Very low	7–8	Much decreased	Much decreased	33% loss in 10 days	,,
Normal on protein-free riboflavin-deficient diet	,,	,,	,, ,,	7–8	,, ,,	,, ,,	Ditto	,,
Normal on 6% protein diet .	High	High	Low	10	Slight increase	Slight increase	Maintained	,,
Normal on 6% protein riboflavin-deficient diet	Low	Low	,,	7–8	,, ,,	Decreased	Slight loss	,,
Normal on riboflavin-deficient diet	High	High	High	4–4·5	,, ,,	,,	,, ,,	,,

both pregnant and tumour-bearing mice. However, the liver weight/total weight ratio increased only in the tumour bearers. In the late stages of tumour growth the ratio returned to normal, and the liver weight and total N fell sharply, but in the pregnant mice the increased liver weight and N were maintained throughout.

The results obtained using the various diets suggest that an increased granule/EPC catalase distribution ratio is a reflection of a mild protein deficiency in the host. However, there seems to be no parallelism between the severity of the protein deficiency and the catalase distribution ratio. In animals maintained on a protein-free diet, the catalase distribution ratio rose rapidly to a value of about 7·5, and the absence of a riboflavin supplement made no detectable difference. However, in the animals kept on the rice-oatmeal diet (diet (3), containing about 6 per cent protein), the catalase distribution ratio reached about 8 when there was no riboflavin supplement, and about 10 when a riboflavin supplement was included in the diet. This difference in distribution ratio occurred largely because there was little or no fall in granule catalase activity in the group receiving riboflavin. In fact a comparison of the results obtained with the various groups of animals shows that in the pattern of the changes in catalase level there were marked similarities between the pregnant mice and the normal mice kept on diet (3) supplemented with riboflavin, and between the tumour-bearing mice and the mice kept on diet (3) unsupplemented with riboflavin. The difference between the pregnant and tumour-bearing mice may therefore be partly explained on the supposition that an agent secreted by the tumours has the properties of a riboflavin antagonist or antimetabolite. However, the mice kept on diet (3) in the absence of riboflavin did not simulate quantitatively the tumour-bearing hosts. Firstly their catalase activities did not reach the low levels seen in the tumour-bearing host, and secondly there was no increase in liver weight, or liver weight/body weight ratio.

The mice kept on the protein-free diet (diet 2) also failed to simulate the tumour-bearing hosts quantitatively. Although the catalase activity in both intracellular fractions rapidly reached the low levels seen in tumour-bearing animals, this was achieved at the expense of a more rapid loss in carcass weight, and an associated rapid loss in liver weight and total N. Also there was no increase in the liver weight/body weight ratio.

The results also show that when animals kept on either diet (2) or (3) were subsequently fed protein, their catalase activity and distribution ratio were rapidly restored to values not far from normal. As mentioned in the introduction Begg and Dickenson (1951) found that force feeding additional protein to tumour-bearing animals did not restore their catalase activity. It is true that those mice kept on diets (2) or (3) with no riboflavin supplement failed to gain body weight when protein was subsequently fed, but this did not prevent a rapid rise in catalase activity. This result agrees with that obtained by feeding a diet containing 20 per cent protein (diet (3) supplemented with 15 per cent of vitamin-free casein) with or without riboflavin. After 7 weeks on the diet the body weight of the riboflavin-deficient group had fallen, and that of the group receiving riboflavin had risen. The only change in catalase levels was a slight fall in the granule activity of the riboflavin-deficient group, with a consequent fall in the granule/EPC ratio to about 4·3. It is perhaps of interest that, as with the mice kept on diet (3) in the presence and absence of riboflavin, a riboflavin supplement

appeared to have the effect of maintaining granule catalase activity, and increasing the granule/EPC distribution ratio compared with the riboflavin-deficient group.

When animals maintained on diet (3) with or without riboflavin were injected with cortisone in sufficient amounts to cause a marked tissue catabolism certain striking changes were seen. Firstly, within a period of 4 days the increased catalase granule/EPC distribution ratio was restored to normal in those animals receiving a riboflavin supplement, and to that characteristic of riboflavin deficiency in those animals not receiving riboflavin. This suggests very strongly that cortisone acts on the permeability of the granules to catalase in the same way as androgenic hormones (Adams, 1960). Table II shows that the increased catalase distribution ratio in pregnant mice was also restored to approximately normal when these animals were given cortisone. In contrast, the increased granule/EPC distribution ratio in tumour bearers did not alter even with the massive amounts of cortisone injected, showing that this action of cortisone is blocked in the tumour-bearing animal.

However, the remaining changes in the cortisone-treated animals, particularly in the riboflavin-deficient group, were strikingly similar to those seen in the tumour bearers. There was an initial increase in liver weight and total N, but both subsequently fell below normal. The rate of loss of body weight was increased to about that seen in tumour bearers, the liver weight/body weight ratio was increased to about the same extent, and total catalase activity fell to the low levels found in tumour bearers. If in fact the action of cortisone on the catalase distribution ratio had been blocked in these cortisone-treated animals they would have simulated the tumour bearers closely in every respect studied. The changes in the group receiving a riboflavin supplement were not so pronounced and the catalase distribution ratio before cortisone was injected exceeded that of tumour bearers. The results also show that when the diets were supplemented with 15 per cent casein, there was an immediate recovery in this group. In the riboflavin-deficient group on the other hand over half the animals showed no response at all to the protein supplement, and the reaction of the remainder was much less than that of the riboflavin-supplemented animals.

Although the results suggest that the tumour-bearing host is in a state of riboflavin deficiency there is other evidence that this may be a simulated rather than an actual state. Evidence against riboflavin deficiency is provided by the normal level of the activity of riboflavin containing enzyme liver xanthine oxidase in the tumour-bearing animal (Greenstein, Jenrette, Mider and White, 1941 ; Greenstein, Jenrette and White, 1941 ; Begg, 1955 ; Burton, 1956). However, this might be explained by the fact that the effect of a riboflavin antagonist or antimetabolite secreted by the tumour, could differ in some respects from that of riboflavin deficiency. The high riboflavin diet coupled with injections of riboflavin had little effect on the catalase activity of the tumour-bearing host. However, there was an indication that the riboflavin-supplemented host lost weight less rapidly. Because the effect was slight, the results were not given previously. In six male mice bearing tumours averaging 2·5 g. the host weight was 19·8 $\pm$ 0·3 g. and in a similar group of riboflavin-treated mice the host weight was 21·2 $\pm$ 0·5 g.

In connection with the possibility that the tumour-bearing animal shows only a simulated riboflavin deficiency, it is of interest that Burch, Hunter, Combs and Schutz (1960) found a reduced capacity for oxidative phosphrylation in

mitochondria prepared from the livers of riboflavin-deficient rats. It seems probable that this is at least one factor in the lowered utilisation of protein caused by riboflavin deficiency (Sure and Dichek, 1941 ; Sure, 1941). Possibly therefore the tumour agent is an inhibitor of oxidative phosphorylation rather than a direct riboflavin antagonist. This point will be investigated in further studies.

The present experiments show a fall in total catalase with protein deprivation or depletion, but in neither case was there any increase in the liver weight/body weight ratio. The diet containing 6 per cent of protein together with cortisone injections resulted in decreases in total catalase activity as great as those seen in mice fed on protein-free diet, and this regime also resulted in an increase in the liver weight/body weight ratio. The animals fed on 6 per cent protein diet and injected with cortisone failed, however, to simulate the tumour bearers in two respects. Firstly, they lost body weight more slowly, and secondly, their catalase granule/EPC distribution ratios were too high. The imposition of a state of riboflavin deficiency in addition to the low protein-cortisone regime increased their rate of loss of body weight and reduced the granule/EPC distribution ratio to that found in tumour bearers. Further, it was found that injection of cortisone into tumour bearers did not further depress their total catalase.

It is suggested therefore that the situation in the tumour-bearing host may be explained in the following way. Firstly, the animals behave as though they were being maintained on a low protein diet, and in this respect simulate the pregnant animal. However, the pregnant animal was able, in the present studies, to support the growth of additional tissue at twice the rate of tumour growth, and to maintain or increase its carcass weight. The situation in the pregnant animal seems therefore to be one of protein "stress" produced by simple competition between maternal and foetal tissue for dietary nitrogen. In the tumour bearer there appears to be a similar competition, but in addition a secretion by the tumour of an agent or agents which

(*a*) Block the action of adrenal hormones on the permeability of intracellular granules, and possibly on other organs.

(*b*) Induce a hypersecretion of adrenal steroids to many times the normal rate, possibly as a result of (*a*).

(*c*) Act to cause or simulate a riboflavin deficiency.

Thus the action of tumour homogenate injections in increasing the catalase granule/EPC distribution ratio in female mice would be due, as previously supposed (Adams, 1951, 1959), to the blocking of the effects of cortisone on granule permeability by an agent present in the tumour. The rise in this ratio in the tumour-bearing host could similarly be explained by the effect of tumour agents on the action of endogenous adrenal steroids. With regard to the fall in catalase activity, it is not necessary to assume that tumour agents directly depress total catalase activity in the tumour-bearing host. The fall could be due to mechanisms (*b*) and/or (*c*) and be secondary to impairment of N retention. It is also of interest that although androgens injected in sufficiently high doses caused immediate depressions in catalase activity superimposed on the alteration in intracellular distribution (Adams, 1960), cortisone in similar doses (Fig. 9) may effect the catalase distribution ratio before depressing catalase activity to any extent. The results suggest that the lowered catalase activity produced by cortisone injection is due to the induced tissue catabolism.

UMI

There are other general points arising from the hypothesis. Firstly, it would seem to provide an explanation of the " nitrogen trap "—not by suggesting that tumours have any unique power of nitrogen uptake, but that the tumour secretes agents which interfere with the ability of host tissues to retain nitrogen. Secondly, it enables an explanation to be provided of the wide variations in the effects of tumours on, for example, the body weight of the host. In the extremes a rapidly growing tumour producing large quantities of agents might be expected to have the most severe effects on the host, while a slow growing tumour producing small quantities of agents might affect its host only slightly.

Thirdly, the results have shown that cortisone-treated mice maintained on a low protein- and riboflavin-deficient diet begin to die in an emaciated state within 3 weeks, and the cause of death presumably is an inability to maintain the levels of essential proteins. As already pointed out, this time of 3 weeks is about that in which a subcutaneously implanted Sarcoma 37 begins to kill its host. Clearly, however, the dietary regime could not simulate death due to metastatic involvement of vital organs, and death from such a cause may often precede what Begg (1958) has called the " metabolic death " of the host.

At present virtually nothing is known about the significance of these tumour agents, but it seems possible that the suggested actions of the secreted agents may assist tumour growth. If the host is largely unable to utilise dietary nitrogen this presumably means that competition for the available material is weighted in favour of the tumour. In this connection there seems no difficulty in explaining why the tumour is not itself affected by, for example, an increased cortisone secretion. Roberts (1953) showed that tissue proteins labilised by pituitary-adrenal cortical stimulation may be utilised for liver regeneration. He suggested that the primary effect of adrenal cortical secretion involves an accelerated mobilisation of protein in the form of plasma proteins, and that the end result locally may be anabolism or catabolism depending on the tissue requirement for protein at the time. It seems possible therefore that the induction of an adrenal hypersection by the tumour may be a method of rapidly providing the building blocks for tumour growth.

One important question is, what would be the effect on the tumour and on the host of preventing the action of the secreted agents. At present this cannot be done completely, because of our ignorance of the primary cause of the simulated riboflavin deficiency in the host. However, a combination of such procedures as adrenalectomy with maintenance therapy, a high protein and high riboflavin diet, and treatment with an anabolic androgen might be worth considering. It does not seem unreasonable to hope that some retardation of the rate of tumour growth coupled with prolongation of the life of the host and protection from wasting might be achieved by proceeding along these lines.

SUMMARY

(1) The effect of the growth of Sarcoma 37 on the total catalase activity, body weight, liver weight and total nitrogen of host mice is closely simulated by maintaining normal mice on a diet containing 6 per cent of protein and deficient in riboflavin, and injecting them daily with 1·5 mg. of cortisone.

(2) When cortisone is injected into tumour-bearing mice, protein deficient mice or pregnant mice, all of which have a raised catalase granule/EPC distri-

bution ratio, the ratio is restored to normal in all but the tumour-bearing group.

(3) It is suggested that tumours release into circulation agents which have the properties of a riboflavin antagonist or antimetabolite, block the effect of adrenal hormones on some target tissues, and induce a marked adrenal hyperfunction. As a result the tumour-bearing host is largely unable to utilise dietary nitrogen.

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Postscript

After this manuscript was completed, a paper by Hilf, Burnett and Borman (1960) reached me, which on the basis of thymus and adrenal weights and adrenal and plasma corticosterone levels in mice, suggested that a growing tumour (Sarcoma 180) induces an adrenal response resembling that resulting from a non-specific chronic stress situation.

REFERENCES

Adams, D. H.—(1950) *Brit. J. Cancer*, **4**, 183.—(1951) *Ibid.*, **5**, 409.—(1952) *Biochem. J.*, **50**, 486.—(1955) *Ibid.*, **60**, 586.—(1959) *Brit. J. Cancer*, **13**, 704.—(1960) *Biochem. J.*, **74**, 141.—(1961) *Brit. J. Cancer*, **15**, 386.

Idem and Burgess, E. A.—(1957) *Ibid.*, **11**, 310.—(1959*a*) *Biochem. J.*, **11**, 310.—(1959*b*) *Enzymologia*, **20**, 341.

Allison, J. B., Bernstein, E. H. and Babson, A.—(1954) *Fed. Proc.*, **13**, 174.

Appleman, D., Skavinski, E. R. and Stein, A. M.—(1950) *Cancer Res.*, **10**, 498.

Babson, A. L. and Winnick, T.—(1954) *Ibid.*, **14**, 606.

Begg, R. W.—(1954) *Proc. Amer. Ass. Cancer Res.*, **1**, 4.—(1955) *Proc. Canad. Cancer Res. Conf.* for 1954, p. 237.—(1958) *Advanc. Cancer Res.*, **5**, 1.

Idem and Dickinson, T. E.—(1951) *Cancer Res.*, **11**, 409.

Iidem and White, A. V.—(1953) *Canad. J. med. Sci.*, **31**, 307.

Bodansky, O. and Scholler, J.—(1956) *Cancer Res.*, **16**, 894.

Burch, H. B., Hunter, F. E., Combs, A. M. and Schutz, B. A.—(1960) *J. biol. Chem.*, **235**, 1540.

Burton, A. F.—(1956) M.Sc. Thesis, University of Western Ontario.

Chance, B.—(1950) *Biochem. J.*, **46**, 387.

Czackes, J. W. and Guggenheim, K.—(1946) *J. biol. Chem.*, **162**, 267.

Finlayson, J. S., Forsberg, A. and Dreyfus, G.—(1959) *Experientia*, **15**, 107.

Greenstein, J. P.—(1954) ' Biochemistry of Cancer '. New York (Academic Press).

Idem and Andervont, H. B.—(1942) *J. nat. Cancer Inst.*, **2**, 345.—(1943) *Ibid.*, **4**, 283.

Idem, Jenrette, W. V. and White, J.—(1941) *Ibid.*, **2**, 17.

Idem, Jenrette, W. V., Mider, G. B. and White, J.—(1941) *Ibid.* **1**, 687.

Hilf, R., Burnett, F. F. and Borman, A.—(1960) *Cancer Res.*, **20**, 1389.

Hubbel, R. B., Mendel, L. B. and Wakeman, A. J.—(1937) *J. Nutr.*, **14**, 273.

Kaunitz, H., Weisinger, H., Blodi, F. C., Johnson, R. E. and Slanetz, C. A.—(1954) *Ibid.*, **52**, 467.

Kennedy, G. C. and Pearce, W. M.—(1958) *J. Endocrin.*, **17**, 149.

LePage, G. A. and Greenlees, J.—(1955) *Cancer Res.*, **15**, 256.

Idem and Henderson, J. F.—(1960) *Exp. Tumour Res.*, **1**, 441.

Idem, Potter, V. R., Busch, H., Heidelburger, C. and Hurlbert, R. B.—(1952) *Cancer Res.*, **12**, 153.

Long, C. N. H.—(1942) *Endocrinology*, **30**, 870.

UMI

Masayama, T. and Yokoyama, T.—(1939) *Gann*, **33**, 214.
Mider, G. B., Tesluk, H. and Morton, J. J.—(1948) *Acta Un. int. Cancr.*, **6**, 409.
Norberg, E. and Greenberg, D. M.—(1951) *Cancer*, **4**, 383.
Paschkis, K. E. and Cantarow, A.—(1958) *Cancer Res.*, **18**, 1060.
Iidem and Stasney, J.—(1956) *Proc. Amer. Ass. Cancer Res.*, **2**, 138.
Iidem and Hobbs, J. H.—(1955) *Cancer Res.*, **15**, 579.
Poo, L. J., Lew, W. and Addis, T.—(1939) *J. biol. Chem.*, **128**, 69.
Poulton, B. R. and Reece, R. P.—(1957) *Endocrinology*, **61**, 217.
Roberts, S.—(1953) *J. biol. Chem.*, **200**, 77.
Robertson, W. van B. and Kahler, H.—(1942) *J. nat. Cancer Inst.*, **2**, 595.
Seegers, W. H.—(1937) *Amer. J. Physiol.*, **119**, 474.
Sherman, C. D., Morton, J. J. and Mider, G. B.—(1950) *Cancer Res.*, **10**, 374.
Straube, R. L. and Hill, M. S.—(1956) *Proc. Amer. Ass. Cancer Res.*, **2**, 150.
Sure, B.—(1941) *J. Nutr.*, **22**, 295.
Idem and Dichek, M.—(1941) *Ibid.*, **21**, 453.
Vivanco, F.—(1935) *Naturwissenschaften*, **23**, 306.
Weil-Malherbe, H. and Schade, R.—(1948) *Biochem. J.*, **43**, 118.
White, F. R. and Belkin, M.—(1945) *J. nat. Cancer Inst.*, **5**, 261.
Williams, R. B.—(1951) *Milit. Surg.*, **109**, 435.
Yeakel, E. H.—(1948) *Cancer Res.*, **8**, 392.
Idem and Tobias, G. L.—(1951) *Ibid.*, **11**, 830.

TUMOUR INCIDENCE AND TUMOUR-FREE SUBLINES IN BR6 MICE

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In 1945 Foulds mated some C57/BL female mice with RIII males. Some of the progeny of these matings developed mammary tumours and the sixth female to do so and a male of the same litter constituted the parents of the BR6 strain which has been maintained by brother-sister mating ever since.

Foulds supposed that the BR6 strain harbours the mammary tumour virus derived from its RIII male progenitor since the virus was demonstrable in a similar C57 × RIII hybrid bearing mammary tumours (Foulds, 1949*a*). The tumour incidence in the strain was high from the start (Foulds, 1949*a*). The tumours are influenced greatly by hormonal conditions; many of them in the original hybrids were readily transplantable into female recipients of the same genetic constitution or into males treated with oestrogen but grew slowly after a long latent period, or not at all, in males (Foulds, 1947), though this sex factor in transplantation tended to be lost in subsequent generations (Foulds, 1949*b*). The most striking characteristic of the tumours in the BR6 line was their pregnancy dependence. The tumours almost invariably first appeared during pregnancy, regressed after parturition, but reappeared in the next pregnancy to grow rather more and regress rather less at the next parturition. This sequence was repeated in subsequent pregnancies with more growth and less regression until eventually the tumour or tumours grew whether the female were pregnant or not (Foulds, 1949*c*). These observations led Foulds to develop his concept of tumour progression (1949*c*; 1954). In 1956 he published an extended analysis of the histological characteristics of the BR6 tumours.

When Dr. Foulds transferred to the Chester Beatty Research Institute in 1950 he left two litters of the 14th inbred generation of the BR6 mice with us and the Mill Hill colony of the strain, derived from these, has been maintained for a further 40 generations. The incidence of tumours during the first 33 of these 40 generations is reported here together with the appearance of sublines free from tumours.

MATERIALS AND METHODS

Maintenance of the colony

Originally each weaned litter was housed in a single cage and the only segregation was of females for delivery and suckling. First litters only from each female were kept and as the colony became established no further litters were kept once the mice in the succeeding generation totalled 100. From the 41st generation

this system was modified : pairs from each first litter were segregated to maintain the colony and subsequent litters were kept to provide animals for experiment.

The mice have been fed successively on various pelletted diets : Rowett Institute 1936 Formula ; Rowett Institute No. 86 ; M.R.C. 41B ; Glaxo GR2 and, at present, Glaxo GR25.

Tumour records

The animals were inspected weekly and all tumours measured by caliper in two diameters. The time of tumour appearance was recorded in relation to age in months and parity, thus 10/4 would indicate that the tumour first appeared when the mouse was 10 months old and had already had 4 litters. Pregnancies and littering dates were also recorded.

Histology

We have not been particularly interested in this matter but sections have been taken of a representative selection of the tumours and show no particular differences from those described by Foulds (1956).

OBSERVATIONS

Time of tumour appearance

Parous mice.—In all there were 514 parous mice with tumours in generations 14–47. The modes for the first appearance of these tumours in generations 14–22 (209 mice), 25–36 (205 mice), and 37–47 (100 mice) were the same : during the 4th pregnancy at the age of 6 months (Table I), though these figures conceal a

TABLE I.—*Frequency of Tumour Appearance in Relation to Age and Parity*

	Age (months)										
Parity	4	5	6	7	8	9	10	11	12	>12	Totals
1	6	8	4	3	2	1	—	1	—	4	29
2	22	31	39	16	4	3	—	—	—	2	117
3	5	24	43	35	17	8	5	3	2	6	148
4	3	3	24	24	25	9	2	3	—	3	96
5	—	1	10	13	15	7	2	2	3	1	54
6	—	—	1	8	9	10	4	2	—	7	41
7	—	1	—	3	2	8	2	2	1	4	23
8	—	—	—	—	1	1	—	—	—	—	2
9	—	—	—	—	2	—	—	1	—	—	3
10	—	—	—	—	—	—	—	—	—	1	1
Totals	36	68	121	102	77	47	15	14	6	28	514

slight tendency for tumours to appear rather later in the most recent generations, apparently because of less frequent pregnancies. These data were arranged in the form of cumulative percentages (Table II) to give a rough index for judging the probability that an animal dying without a tumour would have developed one had it lived longer or had more litters. For a mouse dying when 9 months old and having had 4 litters, for example, the odds against it developing a tumour had it lived longer would be of the order of 100 : 30. This figure is only a rough one however, as the table takes no account of mortality from other causes.

TABLE II.—*Data of Table I Expressed as Cumulative Percentages*

Parity	Age (months) 4	5	6	7	8	9	10	11	12	>12
1	1	3	4	4	4	5	5	5	5	6
2	5	13	21	25	26	27	27	27	27	28
3	6	19	35	46	50	51	54	54	55	57
4	7	20	41	56	66	70	71	73	73	76
5	7	20	43	61	73	79	81	82	83	86
6	7	20	44	63	77	84	87	89	90	94
7	7	20	44	64	78	87	90	92	94	99
8	7	20	44	64	78	87	90	93	94	99
9	7	20	44	64	79	88	91	93	95	100
10	7	20	44	64	79	88	91	93	95	100

Virgin mice.—Tumours have not developed in virgin females younger than 10 months of age.

Tumour incidence

Parous females.—All mice in generations 14–19 having two or more litters developed tumours but the tumour incidence fell rapidly in succeeding generations and was only 48 per cent in generation 26. Inspection of the records showed that all the 26 tumour-free females in this generation had a common tumour-free ancestor in generation 20 and that none of the 114 female descendants of this particular mouse had developed a tumour although 78 of them had borne 2 or more litters. Five of such tumour-free sublines have cropped up and all the mice in these, apart from the 5 initiators of the sublines, are omitted from Table III. This table shows that otherwise only 29 out of 545 parous females failed to develop tumours, an overall tumour incidence of 95 per cent.

TABLE III.—*Tumour Incidence in Parous BR6 Mice, Omitting Those Bearing Less Than 2 Litters and the Tumour-free Sublines*

Generations	Number of parous females	Number with tumours	Tumour incidence (%)	Age /parity of tumour-free females: No progeny	Tumour-bearing progeny	Tumour-free progeny
14–19	126	126	100	—	—	—
20–24	122	116	95	9/5	9/2	18/8
					26/6	16/9
						22/9
25–29	73	69	95	10/3	7/5	—
				8/5	—	—
				25/10	—	—
30–34	92	88	96	7/3	18/7	23/10
					31/9	—
35–39	66	59	89	15/3	4/2	15/7
				7/4	15/2	—
				18/5	17/4	—
40–47	66	58	88	16/2	5/2	—
				5/3	6/3	—
				7/6	7/4	—
				9/9	22/7	—
Totals	545	516	95	12	12	5

Virgin females.—Tumours appeared in 26 virgin females out of 56 (46 per cent) aged 10 months or more. Most of these tumours (18/28) appeared in the mice that died when 10–19 months old but some (9/28) still developed in those dying when 20–38 months old. The higher incidence in the younger mice presumably merely expresses the fact that mice with tumours do not survive.

Males.—No mammary tumours have been seen in male mice.

Pregnancy dependence

This characteristic of the tumours has been maintained until now. Some tumours appear to be independent from their first appearance but our records are not detailed enough for us to be absolutely sure of this. Some of these apparently unresponsive tumours have developed in old mice and resemble those that appear in virgin mice of this age. Even if all the remaining tumours are truly unresponsive they would constitute a small minority of the total number of tumours.

Tumour-free sublines

Occurrence.—Five of the 545 female mice with more than 2 litters had no tumours and had progeny which were free of tumours—an incidence quite out of keeping with any known mutation rate and which might have been even higher had 12 similar tumour-free mice had progeny. In addition there were 12 tumour-free mice which had progeny with a normal incidence of tumours.

A peculiar feature of the progenitors of the 5 tumour-free sublines was that they all had normal tumour-bearing sisters (Table IV) and these, in the few instances where they had progeny, had tumour-bearing descendants.

TABLE IV.—*Mice Giving Rise to Tumour-free Sublines and Tumour Appearance in Their Sisters*

Generation and litter	Tumour-free females		Tumour-bearing females	
	Mouse Number	Age/parity at death	Mouse Number	Age/parity at tumour appearance
20j	67	18/8	66	7/3
24j	55	16/9	56	8/9
	58	22/9	57	8/9
33n	48	23/10	47	6/2
			49	7/2
38r	83	15/7	82	7/4

TABLE V.—*Number of Mice in Tumour-free Sublines*

Original tumour-free female	Parous-female descendants	
	Number of generations bred	Total number of mice
20/67	7	78
24/55	1	4
24/58	23*	167
33/48	6	41
38/83	3	7

* This subline still being maintained.

The tumour-free sublines were usually bred for several generations and Table V lists the total number of females with more than 2 litters in each line. Most of these mice (227 out of 297) were also more than 10 months old when they died or were killed.

Histocompatability.—Had the differentiation of the tumour-free sublines been a genetic change then skin grafts between the sublines and the parent stock would be expected to fail. In the only subline that has been maintained, skin was successfully grafted between this line (BR6/NT) and the parent stock (BR6/T) as shown by the results summarized in Table VI. When these experiments were

TABLE VI.—*Skin Grafting Experiments*

Type of graft	Strain of donor	Strain of recipient	Successful grafts
Orthotopic grafts of adult skin in adult mice	BR6/T	BR6/T	4/6
	BR6/T	BR6/NT	3/4
	BR6/NT	BR6/T	3/4
Foetal skin grafted subcutaneously in adult mice	BR6/T	BR6/T	9/9
	BR6/NT	BR6/NT	7/7
	BR6/T	BR6/NT	7/7
	BR6/NT	BR6/T	9/9
	BR6/T	C3H	0/3
	BR6/NT	C3H	0/5
	BR6/NT	C57	0/9

done, the subline had been separated from the parent stock for 25 generations.

Tumour transplantation.—In view of this finding, it was surprizing that tumour transplants from the parent stock rarely grew in the subline. In these experiments (Table VII) tumours in the late stages of growth, when they were no longer in-

TABLE VII.—*Transplantation of Tumours no Longer Pregnancy-dependent into Mice of Tumour-free Subline*

	Successful transplantation in			
	Tumour-prone line		Tumour-free line	
Experiment	Males	Females	Males	Females
1	4/4	4/4	0/4	1/4
2	3/3	3/3	0/3	1/3
3	2/2	2/2	0/2	0/2

fluenced by pregnancy, were transplanted. It is probably significant that the only two successful transplantations into the non-tumour mice were in females—the original BR6 tumour had shown such a sex preference in transplantation (Foulds, 1947, 1949*b*) but this was soon lost under normal conditions for transplantation. These two transplants grew more slowly and after a much longer latent period than the transplants in the parent stock.

Cross-suckling experiments.—The successful transplantation of skin and largely unsuccessful transplantation of tumours in the tumour-free sublines suggests

some specific antagonism to tumours. Preliminary studies by cross-suckling indicate that if this antagonism is exerted against the mammary-tumour virus, then it is not an absolute one. The preliminary results (Table VIII) suggest the following

TABLE VIII.—*Cross-suckling Experiments*

Strain of mouse		Number of generations bred	Number of ≥ 2-parous females	Number of ≥ 2-parous females with		
Sucklings	Foster mother			Dependent tumours	Independent tumours	No tumours
BR6/T	C57	2	10	0	0	10
BR6/T	C3H	2	11	1	4	6
BR6/NT	C3H	2	12	3*	6	3
BR6/NT	BR6/T	3	15	8	4	3

* One tumour developed in a pregnant mouse which died before pregnancy-dependence could be fully characterized.

conclusions. First, if females of tumour-prone lines are deprived of virus by being suckled on C57/BL mothers, then they do not develop any mammary tumours. Secondly, if these mice are deprived of their own virus and virus from C3H mothers is substituted then the tumours they develop resemble C3H tumours in their growth which tends to be uninfluenced by pregnancy. Thirdly, mice of the tumour-free sublines provided with C3H virus usually grow tumours that are not pregnancy-dependent but grow mostly pregnancy-dependent tumours when provided with virus from the tumour-prone BR6 mice. These results suggest that the BR6 virus and the C3H virus have different properties and that pregnancy-dependence in tumour growth is a result of both the type of virus and of its environment.

COMMENT

Medical research is sometimes criticized for studying disease rather then health. P.C.W. certainly merited such criticism when he first became aware that a tumour-free subline was swamping the precious tumour-bearing strain. His immediate reaction, immediately carried out, was to kill all mice of the offending subline. His next reaction was a horrified realization of what he had done. Fortunately other tumour-free sublines arose and the mistake could be rectified. The question why the mice in these sublines do not develop tumours is obviously as, or more, interesting than the question why the mice of the parent strain do develop tumours.

The occurrence of the sublines was as unexpected as it is unexplained and we do not propose to speculate on its significance until more facts are available.

SUMMARY

A colony of BR6 mice, derived from that established by Foulds (1947), has been maintained by brother-sister mating for 40 generations.

The incidence of mammary tumours in the 545 females whose mothers had tumours and who themselves had two or more litters was 95 per cent.

Five of the 29 mice comprising the tumour-free 5 per cent, when mated with their brothers, gave rise to tumour-free sublines.

One of these sublines has been maintained for 25 generations separated from the parent strain.

Skin can be grafted from parent strain into subline but tumours are only exceptionally transplantable in the same way.

We are very grateful to Messrs. L. E. G. Miall, B. F. Wise and D. E. Brown for looking after the colony and keeping the tumour records.

REFERENCES

FOULDS, L.—(1947) *Brit. J. Cancer*, **1**, 362.—(1949*a*) *Ibid.*, **3**, 230.—(1949*b*) *Ibid.*, **3**, 240.—(1949*c*) *Ibid.*, **3**, 345.—(1954) *Cancer Res.*, **14**, 327.—(1956) *J. nat. Cancer Inst.*, **17**, 701.

CHEMICAL INDUCTION OF BREAST TUMOURS IN MICE OF THE C57Bl STRAIN. THE INFLUENCE OF PSEUDOPREGNANCY, PREGNANCY AND LACTATION ON INDUCTION BY METHYLCHOLANTHRENE

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THE induction of breast tumours by cutaneous application of methylcholanthrene (MC) has now been studied in mice of several strains devoid of the mammary tumour agent, under different hormonal influences. The most extensive studies have been made on the IF strain, which appears to be particularly sensitive. It has been found that the incidence of breast tumours resulting from a standard carcinogen treatment of female IF mice varies enormously with the hormonal status of the mice. Virgin females yield a high incidence, but ovariectomised females treated with oestrogen fail to yield tumours unless progesterone is also given (Bonser, 1954; Jull, 1954). It is believed that high levels of progesterone are normally acting in IF virgin females which account for the high incidence of breast tumours induced in them. Females kept with vasectomised males to produce a state of pseudopregnancy are even more sensitive than virgins (Marchant, 1958). Forced breeding IF females, which have their young removed to prevent suckling, produce a high incidence of tumours similar to that of virgins (Marchant, 1958). Breeding, with lactation during the period of carcinogen administration, has an extraordinary power to prevent breast tumours in the IF strain (Marchant, 1955 and 1958). A similar protective effect has recently been obtained in Sprague-Dawley rats (Dao, Bock and Greiner, 1960). Excision of nipples on one side of an IF mouse, which is then allowed to breed and lactate during MC administration, results in localisation of breast tumours on the non-lactating side (Marchant, 1959).

Less extensive studies of other agent-free mouse strains have recently been made. Biancifiori, Bonser and Caschera (1959) found that virgin BALB/c mice were not sensitive to MC treatment, but 44 per cent of females caged with vasectomised males to induce pseudopregnancy developed breast tumours. Ranadive, Hakim and Kharkar (1960) found that MC treatment failed to produce breast tumours in virgin females of the dba and L(P) strains, but there was a high incidence in breeders and in pseudopregnant females, particularly if the pseudopregnancy was brought about by ligating the fallopian tubes and mating to normal males.

The present report describes the induction of breast tumours by MC in mice of the C57Bl strain—a strain which, in such comparative studies as have been made, seems to be particularly resistant to breast tumour induction by MC (Kirschbaum, Williams and Bittner, 1946; Dmochowski and Orr, 1949*a*; Ranadive and Hakim, 1958). It is also known to be relatively resistant to breast tumour induction when infected with mammary tumour agent (Dmochowski and

Orr, 1949*b*, Mühlbock, 1956). The response of this strain to MC has been tested to see whether those factors which influence the induction of breast tumours in the responsive IF strain would similarly influence the resistant C57Bl strain.

MATERIALS AND METHODS

The C57Bl mice used in this study were descended from a pair obtained in 1949 from L. Dmochowski, then in Leeds. They are maintained by brother-sister matings. In our laboratories, spontaneous breast tumours have not been found in old virgins or breeders. Early attempts to induce breast tumours, with a dosage of MC similar to that which induced a high incidence in IF virgins, failed to induce any in C57Bl virgins.

Mice were housed in metal boxes of two sizes. The larger measured $20 \times 28 \times 11$ cm. and the smaller $11 \times 28 \times 11$ cm. A cube diet was given with water *ad libitum*.

Carcinogen treatment.—All groups of mice used in this study received cutaneous applications of 0·5 ml. of olive oil containing 0·5 per cent (2·5 mg.) 20-methylcholanthrene (MC). The applications were made at fortnightly intervals and each dose was spread over the body surface. Eight applications in all were made to all groups of mice except virgin females, in which they were continued throughout life.

Five groups of mice were maintained :—

Group I (14 mice). Young adult virgin females were maintained in large boxes, between 4 and 6 mice per box. Fortnightly applications of MC solution were given throughout life.

Group II (21 mice). Young adult female mice were maintained in a pseudo-pregnant state by caging them in groups of 4 together with 2 vasectomised males in each large box. Two months later 8 fortnightly applications of MC were begun.

Group III (22 mice) Forced-breeding mice were kept in small boxes, 2 females being mated with 1 normal male. They were examined frequently for litters which were noted and removed when discovered. Eight fortnightly MC applications were begun after the birth of the first litter.

Group IV (20 mice) Normal breeding mice were kept under conditions similar to those of Group III but these mice were allowed to rear their litters. MC applications were begun after the initiation of the first lactation.

Group V (20 mice) Unilateral nipple excision was performed on this group a few days before mating. They were then kept as normal breeders under similar conditions to Group IV except that half of the babies in each litter were removed.

Mice were kept as long as their condition remained good. They were examined regularly for tumours and, at post mortem, breast tumour material was fixed in formolsaline and sectioned for histological examination. In many cases whole-mount preparations of non-tumourous breast tissue were made after fixation in Bouin's fluid and staining with Mayer's haemalum. Samples of breast tissue from untreated C57Bl mice were examined for comparison.

RESULTS

Breeding performance.—The MC treatment did not adversely affect the breeding performance of the mice. All breeding mice had one litter before MC applications were begun. Thereafter the mean number of litters born to mice in each

group was as follows: Forced-breeders — 6; normal breeders — 3·8; normal breeders with unilateral nipple excision — 3·9.

Survival.—The survival of the mice was determined in general by the appearance of skin tumours, breast tumours, or (in 12 animals) lymphomatosis.

The survival is shown, together with the appearance of breast tumours, in Fig. 1. The continuous MC treatment of the virgin group led to a greater deterioration of condition than the limited treatment of other groups. All developed skin tumours and it was necessary to terminate their lives after 41 weeks.

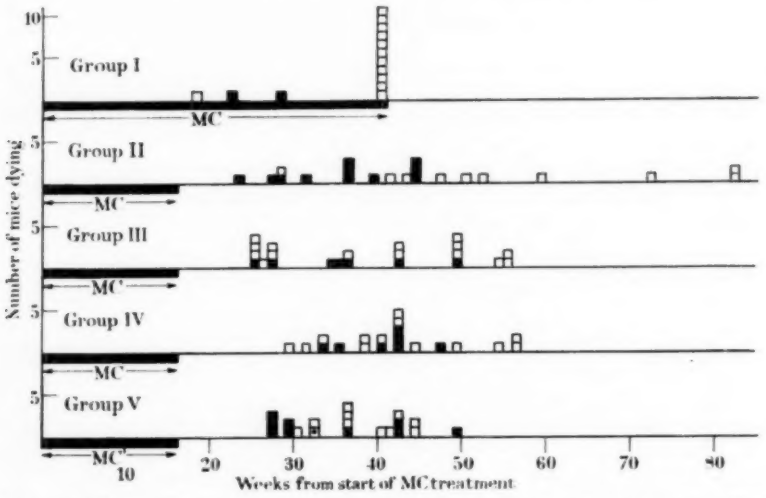


FIG. 1.—Incidence of breast tumours in $C_{57}Bl$ mice treated with methylcholanthrene (MC).

Group I	Virgin (14 mice)	□ Dead without breast tumour
Group II	Pseudopregnant (21 mice)	■ Dead with breast tumour
Group III	Forced breeders (22 mice)	
Group IV	Normal breeders (20 mice)	
Group V	Normal breeders with half nipples excised (20 mice)	■ Tumour on non-lactating side ⊡ Tumour on lactating side

Skin tumours.—These were the most common tumours, occurring in 64 of the 97 animals. They generally arose as papillomas going on to squamous carcinomas, and necessitated killing the animals from the 26th week onwards. They also occurred in the longest survivors at 83 weeks. Many animals bore multiple skin tumours. In general they grew much more slowly than breast tumours.

Breast tumours

Breast tumours occurred in all groups of animals from the 23rd week onwards. The incidence and mean latent period of breast tumours in the different groups of animals is given in Table I.

Multiple breast tumours (maximum 3) were found in 4 of the forced-breeders, 2 normal breeders and 1 breeder with unilateral nipple excision.

TABLE I.—*Incidence and Mean Latent Period of Breast Tumours in C57Bl Mice Treated with Methylcholanthrene (MC)*

Group	Treatment	Breast tumours per cent		Latent period (weeks) Mean	Range
I Virgins	MC throughout life	2/14	14	26	23–29
II Pseudopregnant	8 MC	11*/21	52	36	24–45
III Forced-breeders	8 MC	7/22	31	36	26–50
IV Normal breeders	8 MC	7/20	35	41	34–48
V Normal breeders with unilateral nipple excision	8 MC Lactating side	1/20	5	33	33
	Excised side	9/20	45	35	28–50

* Two were sarcomas.

The survival of the virgin group of animals was not strictly comparable with that of the other groups, but it is considered unlikely that any more tumours would have developed in them for the following reasons. The animals of this group which bore breast tumours developed them early, compared with other groups, and one would have expected other animals of the group to do likewise. Also, very few mice in any group surviving more than 45 weeks developed breast tumours, so almost all tumours would have been detectable by 41 weeks, which was the time the virgin experiment was terminated.

Histology of breast tumours

The great majority of tumours were adenocarcinomata. Squamous metaplasia was common in all groups of animals. Less frequently eosinophilic secretion was seen. A few tumours were of the papillary cystic type and in a very small number of others the stromal fibroblastic cells predominated. Two of the tumours in Group II (pseudopregnant) were found to be sarcomas. In some of the mice with multiple tumours, the individual tumours of the same mouse were dissimilar.

Structure of the non-tumorous breasts

Breast tissue of young untreated virgin C57Bl mice consists of a simple duct system with side-branches, some of which end in buds (Fig. 2). There are no acini present. In older virgins the buds develop a little further into small duct branches, but no acini appear. Vaginal smears of C57Bl virgins show oestrus cycles in which the oestrus phase predominates, being interrupted by a more brief phase of dioestrus on an average every 5 days. When C57Bl females are mated with vasectomised males, they immediately go into a lengthy period of dioestrus only emerging from it into a very brief phase of oestrus about every 2 weeks or so. During this state of pseudopregnancy the breast tissue is stimulated and small groups of acini appear on the fine duct branches (Fig. 4). Immense acinar proliferation and lobule formation occurs during the later part of pregnancy. These regress considerably after weaning.

Methylcholanthrene has been found to produce a proliferation of breast acini (Jull, 1956). However this was not evident after 3 months MC treatment in the virgin C57Bl mouse depicted in Fig. 3. Under the conditions of pseudopregnancy and breeding studied here the breast tissue from the MC-treated mice

was similar to the corresponding untreated mice as far as general development was concerned but, instead of the structure being regular throughout, there were foci of various kinds of hyperplasia. The most frequent type of lesion resembled a cluster of enlarged alveoli (Fig. 5 and 7). Ducts were often enlarged and sometimes congested with concretions. Fewer irregularities were seen in breasts which had lactated than in those which had not. Microscopic tumours can be seen in Fig. 6 and 7 which show breast tissue from breeding mice (Groups III and IV) that did not have palpable breast tumours elsewhere.

DISCUSSION

Although virgin mice of the C57Bl strain do not develop breast tumours when treated with a dose of methylcholanthrene (MC) sufficient to induce breast tumours in 70 to 75 per cent of IF virgin mice, the present experiments show that they can be induced in the following ways. A small incidence can be obtained in virgins by maintaining the MC treatment throughout life, but a much greater incidence can be obtained by keeping the mice under the hormonal conditions of pseudopregnancy or pregnancy during the standard MC treatment. As with the IF strain, the greatest incidence was obtained in the pseudopregnant females kept mated to vasectomised males. It seems clear that the increased oestrogen-progesterone activity associated with pseudopregnancy and pregnancy augments the carcinogenic action of MC on C57Bl mouse breast tissue, just as it does on IF breast tissue.

A notable difference between the IF and C57Bl strains was found in breeding animals. In forced-breeding animals the incidence of tumours found in the two strains was relatively high for each strain. However, when lactation was allowed, the final incidence in the C57Bl strain was not reduced, although the latent period of development was delayed. On the other hand, in the IF strain, lactation during the period of MC treatment completely prevented the appearance of breast tumours. It is known that MC is secreted in the milk but, since there was no

EXPLANATION OF PLATE

Whole mounts of C57Bl female breast tissue. × 12.

FIG. 2.—Untreated virgin, aged 14 weeks. Ducts with no lobules of acini, but some end buds. (In older virgins there is slightly greater development of fine ducts.)

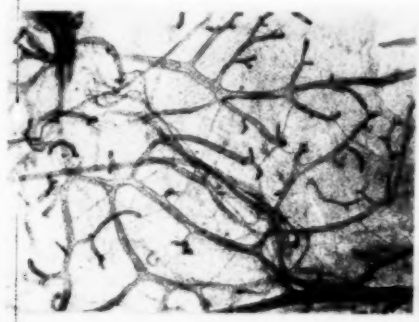
FIG. 3.—Virgin aged 41 weeks. Received 8 fortnightly treatments with MC from 25 to 39 weeks. The breast tissue seems virtually unaffected by the treatment.

FIG. 4.—Pseudopregnant, aged 15 weeks, mated to vasectomised male 3 weeks earlier. There has been some development of fine duct branches with acinar buds.

FIG. 5.—Pseudopregnant (Group II) aged 43 weeks. Mated to vasectomised male at 7 weeks and treated with MC from 15 to 29 weeks. This mouse had a tumour in another breast. There are many foci of irregular development superimposed on a background similar to Fig. 4.

FIG. 6.—Forced breeder (Group III) aged 71 weeks. Mated at 24 weeks; 7 litters from 28 to 53 weeks removed at birth. MC treatment from 29 to 43 weeks. This mouse developed a squamous carcinoma. It had no macroscopic breast tumours, but a number of microscopic foci similar to those illustrated were found. Acinar development was more marked in these animals than in the pseudopregnant group.

FIG. 7.—Normal breeder (Group IV) aged 73 weeks. Mated at 26 weeks; 5 litters suckled between 29 and 54 weeks, MC treatment from 29 to 43 weeks. This mouse developed a squamous carcinoma, but no macroscopic breast tumours. Ducts were dilated and there were foci of dilated acini in addition to normal acini. The microscopic tumour illustrated appeared to consist of dilated acini.



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3

4

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significant difference between the 2 strains in the average number of litters suckled after the beginning of MC administration, it is remarkable that the breast tumour inhibition should be so much greater in the IF strain, which undoubtedly has a greater overall sensitivity to MC carcinogenesis.

The effect of unilateral nipple excision on normal breeding mice was virtually to localise breast tumours on the non-lactating side in both IF and C57Bl strains. This indicates that the delay in tumour appearance in C57Bl breast tissue caused by lactation is a significant one.

The induction of breast tumours in F_1 C57Bl/IF mice by MC is now being studied to see whether they are influenced in the same way as either parent strain.

SUMMARY

Female mice of the C57Bl strain (devoid of mammary tumour agent) received cutaneous applications of 20-methylcholanthrene in olive oil at fortnightly intervals.

This treatment induced breast tumours in only 2 of 14 virgin animals when it was maintained throughout life.

Treatment limited to 8 applications induced tumours in 11 of 21 females kept in a pseudopregnant state by mating with vasectomised males.

Forced-breeding animals, from which litters were removed soon after birth, developed tumours in 7 of 22 animals.

A similar incidence of tumours (7 of 20) occurred in breeding mice which were allowed to lactate, but the latent period of development was increased.

Lactating breeders, with nipples excised on one side to prevent nursing on that side, developed tumours on the excised side in 9 and on the lactating side in 1 of 20 cases.

The majority of mice also developed skin tumours.

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REFERENCES

Biancifiori, C., Bonser, G. M. and Caschera, F.—(1959) *Brit. J. Cancer*, **13**, 662.

Bonser, G. M.—(1954) *J. Path. Bact.*, **68**, 531.

Dao, T. L., Bock, F. G. and Greiner, M. J.—(1960) *J. nat. Cancer Inst.*, **25**, 991.

Dmochowski, L. and Orr, J. W.—(1949*a*) *Brit. J. Cancer*, **3**, 376.—(1949*b*) *Ibid.*, **3**, 520.

Jull, J. W.—(1954) *J. Path. Bact.*, **68**, 547.—(1956) *Acta. Un. int. Cancr.*, **12**, 653.

Kirschbaum, A., Williams, W. L. and Bittner, J. J.—(1946) *Cancer Res.*, **6**, 354.

Marchant, J.—(1955) *J. Path. Bact.*, **70**, 415.—(1958) *Brit. J. Cancer*, **12**, 55.—(1959) *Nature, Lond.*, **183**, 629.

Mühlbock, O.—(1956) *Acta Un. int. Cancr.*, **12**, 665.

Ranadive, K. J. and Hakim, S. A.—(1958) in 'International Symposium on Mammary Cancer,' 1957, ed. L. Severi, Perugia (Division of Cancer Research) p. 441.

Iidem and Kharkar, K. R.—(1960) *Brit. J. Cancer*, **14**, 508.

INCREASE IN MAMMARY CARCINOMA AND ADENOMA AND INCIDENCES OF OTHER TUMOURS IN C_3Hf VIRGIN FEMALES AFTER OVARIECTOMY AND HIGH DOSAGE WITH SOME OESTROGENS

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INCIDENCES of mammary carcinoma and adenoma in C_3Hf virgin females, in those ovariectomised only or given 5 μg. oestrone weekly have been reported (Pullinger, 1959). The present paper records increases in carcinoma and adenoma after ovariectomy and treatment with 10 μg. weekly of oestrone alone and with 10 μg. weekly of a mixture of oestrone, oestradiol-17 β and oestriol. Treatment with 10 μg. oestriol alone gave the same yield of mammary carcinoma as 5 μg. oestrone, namely 2·9 per cent. Tumours that arose in other sites, effects in adrenal glands, results of grafts and the site and age distribution of the mammary carcinomas are included.

METHODS AND MATERIALS

Surplus females of the C_3Hf pure line colony bred for experiment were separated from males and collected in groups at weaning. The origin of the strain, management, methods of treatment and examination have been described (Pullinger and Iversen, 1960; Pullinger, 1960*a*, 1960*b*, 1961). Bilateral ovariectomies were done at 52 to 98 days of age in Experiment I and at 96 to 111 days in Experiment II. The second group was begun with older mice because deaths from urinary calculi and their consequences had been high in the first group in comparison with older and bigger breeding females (Pullinger, 1961). After ovariectomy, weekly treatment with 10 μg. oestrone dissolved in acetone was begun and continued for 60 weeks. A further test which had already been started treating virgin females with 20 μg. oestrone was abandoned on account of their poor condition and high death rate due to urinary calculi. This tendency to calculi in C_3H mice given oestradiol benzoate by subcutaneous injection was reported earlier by Schenken, Burns and McCord (1942), Schenken and Burns (1943).

In Experiment III, 10 μg. weekly of oestriol only was given and in Experiment IV a mixture of 10 μg. weekly of oestrone, 3 parts, oestriol, 3 parts, and oestradiol-17β, 1 part. When it became evident that no urinary calculi were formed when oestriol only was used, a test, in Experiment V, of 200 μg. weekly was begun for comparison of tolerance and tumour induction with other oestrogens.

Grafts from 6 of the mammary carcinomas induced in females and from 4 induced with oestrogen in castrated males in another experiment were made in

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C_3Hf hosts or their F_1 hybrids bred by mating with C_{57} black males. Some doubt was expressed by Foulds (1956) whether secretion of a milk-like substance can occur in mammary tumours in the absence of recent oestrogenic stimulation. One grafted spontaneous mammary tumour that had been growing in an intact male for 3 months was examined for lipoid secretion. Results are recorded later.

Partial ovariectomies were done on 22 virgin female mice by Lipschutz's method (1925). Neonatal gonadectomy was done on 9 female and 14 male mice from 5 to 8 days after birth in an endeavour in the first place to obtain an oestrogen secreting adrenal cortical tumour. Gonads were removed under combined biomethol (0·03 to 0·05 ml. of a 1·5 per cent suspension given intraperitoneally) and open ether anaesthesia.

RESULTS

Experiment I

Of 30 females ovariectomised between 52 and 98 days of age and treated subsequently with 10 μg. oestrone weekly, one was lost and 4 died without tumour before 12 months of age. One died at 292 days from ascending pyelonephritis.

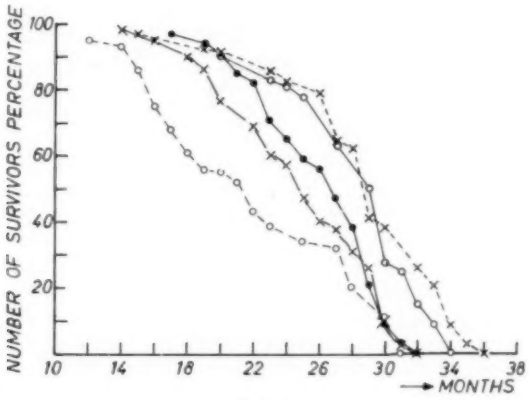

FIG. 1

Ovariectomy only.
Ovariectomy and 5 μg. oestrone.
Ovariectomy and 10 μg. oestrone.
Ovariectomy and 10 μg. oestriol.
Ovariectomy and 10 μg. { oestrone, oestriol, oestradiol.

Twenty-five survived of which 10 were found to have signs of retention or pyelonephritis due to urinary calculi of calcium, ammonium and magnesium phosphates. In comparison with virgin females treated previously with 5 μg. oestrone weekly (Pullinger, 1959) their death rate was increased (Fig. 1). They were poorly and under-weight during the treatment period even in the absence of calculi and infection. Four developed mammary carcinoma, one after 590 μg at 17 months of age, 2 at 22 months and 1 at 28 months. None of these 4 had signs of calculi.

Experiment II

Of 20 mice ovariectomised from 96 to 111 days of age, one died aged 213 days with bilateral pyelonephritis but no tumour. The remaining 19 lived to 12 to 31 months, 2 dying soon after 1 year of age. Seven were found to have urinary calculi at necropsy. Another 7, without signs of calculi, developed mammary carcinoma, one at 16 months after 510 μg. oestrone, one at 17 months after 550 μg. and the remainder after 600 μg. at 21, 22, 23, 27 and 27 months.

In the two experiments combined 11 out of 44 survivors (= 26 per cent) developed mammary carcinoma and one bore 2 primaries at the same time. Age at death and appearance of tumours are given in Table I with survival rates in Fig. 1. These results are in accord with those of Boot and Mühlbock (1956) who showed that when oestrone was added to the drinking water of C_3Hf mice in two concentrations, mammary tumour incidence rose with the higher amount.

TABLE I.—*Incidence of Mammary Carcinoma and Survival Age for C_3Hf Virgin Females After Ovariectomy Alone or Subsequent Treatment with some Oestrogens*

	Age in days at ovariectomy									
	61–65		66–69		52–111		67–70		79–93	
	Ovariectomy only		Oestrone 5 μg. weekly		Oestrone 10 μg. weekly		Oestriol 10 μg. weekly		Oestrone, oestriol and oestradiol 10 μg. weekly	
Age at death in months	Number of mice	Number with carcinoma	Number of mice	Number with carcinoma	Number of mice	Number with carcinoma	Number of mice	Number with carcinoma	Number of mice	Number with carcinoma
12	0	—	0	—	2	—	0	—	0	—
13	0	—	0	—	0	—	0	—	0	—
14	0	—	0	—	1	—	0	—	1	1‡
15	0	—	1	—	3	—	0	—	0	—
16	0	—	0	—	5	1*	0	—	1	—
17	0	—	0	—	3	2†	1	—	0	—
18	0	—	0	—	3	—	0	—	2	1
19	1	—	1	—	2	—	0	—	2	—
20	2	—	1	—	1	—	2	—	4	1
21	0	—	0	—	1	1	2	—	3	1
22	0	—	0	—	4	3	1	—	0	—
23	2	—	2	—	2	1	4	1	4	1
24	1	—	1	—	0	—	2	—	1	1
25	1	—	0	—	1	—	2	—	4	2
26	0	—	1	—	0	—	1	—	2	1
27	5	—	5	—	2	2	3	—	2	—
28	0	—	1	—	5	1	3	—	3	—
29	4	—	7	1	2	—	6	—	2	—
30	7	—	1	—	2	—	4	—	7	1
31	1	—	2	—	5	—	2	—	3	1
32	3	—	1	—	0	—	1	—	1	—
33	2	—	3	—	0	—	0	—	0	—
34	3	—	4	—	0	—	0	—	0	—
35	0	—	2	—	0	—	0	—	0	—
36	0	—	1	—	0	—	0	—	0	—
Totals	32	0	34	1	44	11	34	1	42	11
Percentages	—	0	—	2·9	—	25	—	2·9	—	26·1

No entry = no tumour.
* Total dose = 510 μg. oestrone.
† Total doses = 550 and 590 μg. oestrone.
‡ Total dose = 470 μg. of mixed oestrogens.

In the present tests adenomatous nodules were found in 18 out of a sample of 19 complete mammary glands that were examined (Table II). Development of ducts was more extensive than in mice given 5 μg. oestrone weekly and lobular-alveolar development occurred more often.

TABLE II.—*Adenomatous Nodules After Ovariectomy and Substituted Oestrogens in C_3Hf Virgin Females*

	Number of mice examined	Number with nodules	Percentage with nodules	Total nodules
Ovariectomy only	28	1	3·57	1
Oestrone 5 μg.	31	5	16·1	8
Oestrone 10 μg.	19	18	94·7	29
Oestriol 10 μg.	22	3	13·6	30
Oestrone, Oestriol, Oestradiol } 10 μg.	28	20	71·4	149
Intact	93	27	27·9	—

— = Counts could not be made reliably because not all mammae had involuted.

Eighteen pairs of adrenal glands from Experiments I and II were examined microscopically. In 4 pairs from mice that died before the last treatment with oestrone, cells of type A but not of type B of Woolley and Little (1945) were found in the adrenal cortices and in 3 of them the majority of zona fasciculata cells were almost completely compact. The change to compact is normal in ageing mice of this strain (Pullinger, 1959) and had not been prevented by giving oestrone nor had the development of type A cells. In 2 of these 4 mice mammary carcinoma had already developed. After the end of treatment, from 17 months of age onwards, a few clusters of B cells (mainly pigmented with ceroid) were found in 9 pairs. In the remaining 5 pairs examined, type B cells were hyperplastic and in one mouse aged 23 months with a mammary carcinoma there was capsular infiltration; in another aged 22 months also with a mammary carcinoma there was a macroscopic adrenal cortical carcinoma. This carcinoma was similar to all others seen in this strain except that it contained some acini with eosinophilic PAS positive secretion among spherical groups of anaplastic tumour cells. The uterus and horns were not enlarged or functional in either mouse and the vaginal epithelium was atrophic, indicating a lack of secretion of oestrogen.

Of mice with tumours of other sites there were 2 with hepatoma; one with a wart and one with a squamous carcinoma of skin; 2 with lung adenoma; one with a polymorphic-celled sarcoma; one had a lymphosarcoma and one a macroscopic lymphoma. No enlarged pituitaries were found.

Experiment III. Oestriol 10 μg.

Of 34 virgin females ovariectomised between 57 and 67 days of age and given 10 μg. weekly of oestriol for 60 weeks, all lived to 17 months or more (Fig. 1). One developed a mammary carcinoma at 23 months old (Table I). Three only of a sample of 22 whole mammary glands had adenomas. The total number of adenomas was 30 varying from 3 to 4 and to 23 in individuals. The mouse with 23 had the only mammary carcinoma, also a hepatoma and her salivary gland

was one of 2 of male type among 8 with hepatoma and 7 without that were examined microscopically.

Nineteen pairs of adrenal glands were examined microscopically. All had type A and compact fasciculata cells. Type B cells were found in 17, in 9 of which they were pigmented, in 2 hyperplastic and in 3 had invaded the capsule. None of the invasive type was associated with mammary carcinoma or adenoma. There was one medullary tumour. Of tumours of other sites there were 10 hepatomas, 5 gross lymphomas, one spindle-celled and one osteogenic sarcoma, one squamous carcinoma of the clitoris and one of skin. No pulmonary adenomas were found as compared with 14 in 110 intact virgin females, 2 in 32 ovariectomised only and one in 34 ovariectomised and given 5 μg. oestrone. No urinary calculi were found. No pituitary glands were enlarged.

Experiment IV

Of 44 virgin females ovariectomised between 79 and 93 days of age and treated with 10 μg. weekly of the mixture of three oestrogens, 2 died from intercurrent infections before 12 months of age and neither had calculi or tumours. Eleven of the remaining 42 (= 26 per cent) bore these carcinomas and one had 2 primary growths. Of 28 complete mammae examined by bulk-staining, 20 had adenomatous nodules varying from 1 to 38 per mouse with a total of 169 (Table II). All nipple regions of these mice were more extensively developed than after treatment with oestrone only and 15 were very well developed with widespread lobular-alveolar differentiation which persisted to 26 months of age. Later the glands were involuted. At its greatest development, the lobular-alveolar development was slight in comparison with any fully developed mammae in pregnancy. Thus the cell population that developed and was exposed to oestrogens was far less numerically, nevertheless similar proportions of females bearing carcinoma, 26 per cent and of adenoma, 71·4 per cent, were found as in breeders. Urinary calculi or infection were present in 6, none of which had mammary tumours. The survival rate was better than in the group given 10 μg. of oestrone alone (Fig. 1). The amount of calculus producing oestrogen in the mixture was approximately 5·72 μg. Salivary glands of 4 with hepatoma and of 6 without were female in type. Glomerular linings were female in type in 2 and male in type in 2 with hepatoma and female in 4 and tending to male in 2 out of 6 examined without hepatoma.

Twenty-five pairs of adrenal glands were examined microscopically. Four had type A and compact zona fasciculata cells in the cortex and in one the X-zone persisted; pigmented and non-pigmented type B cells were found in 21 pairs and in 6 of these were hyperplastic. Accessory adrenal cortical compact cells occurred in 2 mice. Five had hepatoma. The salivary glands of 4 with and of 6 without hepatoma were examined microscopically. All ducts were female in type. There were 4 mice with sarcoma, 3 with pulmonary adenoma, 2 with squamous carcinoma of skin and 2 with macroscopic lymphoma. No enlarged pituitaries were found.

Percutaneous application to 154 ovariectomised virgin female mice of oestrogen in amounts of 5 μg. and 10 μg. weekly for 60 weeks was associated with 11 sarcomas as compared with 13 in 110 intact females, a difference without significance. There was a decrease in pulmonary adenoma compared with intact

females and in those given oestriol only, but owing to low spontaneous incidence in this strain and the shorter survival time of the treated mice the result is inconclusive. A significant decrease in hepatoma to the level of breeding females accompanied treatment with 10 μg. oestrone weekly but again the validity of this reduction is questionable on account of their poor condition and reduced survival time. According to Heston, Deringer and Vlahakis (1960) the better nourished, more rapidly growing males develop higher incidences of hepatoma.

It was noticeable that all those virgin females which developed mammary carcinoma were free of urinary calculi and the same was true of the breeders treated previously. However, the tumour incidence was too low to give this observation significance.

Adrenal cortical tumours

Seven macroscopic and 7 microscopic cortical carcinomas have been found in the mature male and female C_3Hf mice that have been gonadectomised for various purposes. Hyperplasia and adenoma of type B cells (according to the description of Woolley and Little, 1945) were far more frequent than carcinoma. In the females evidence of secretion of oestrogen was obtained at latest 8 months after ovariectomy from vaginal cornification tests. The amount was judged from examination of nipple regions to be less than the equivalent of 5 μg. weekly of oestrone (Pullinger, 1959). From similar biological evidence the gross tumours and their first generation grafts were found to be less likely to secrete oestrogen than were hyperplastic type B cells. The impression was gained that actively developing B cells were the oestrogen producers of this strain rather than the typically anaplastic tumour cells. Three only of 7 macroscopic carcinomas, one in a castrate male and 2 in ovariectomised virgin females were associated with evidence in mammary structures of stimulation by oestrogen. However, owing to the slow growth of grafts in gonadectomised (and also in intact) hosts, type B cells were present and hyperplastic in the adrenals of all three and therefore the source of the oestrogenic stimulation was in doubt. Of 9 newly born females ovariectomised from 5 to 8 days old, all were found to be in continuous oestrus or producing non-nucleated squames in excess of polymorphs by 8 months of age. After one year old the previous voluminous vaginal contents became scanty in all 9 in succession and at the present time, 21 months of age, relatively few oestrus cells can be found in 2 out of 5 still living. Of the 4 dead females, the adrenals of one were replaced by lymphoma cells. In the other 3, type B cells mainly laden with ceroid were hyperplastic and in one had infiltrated the capsule. It seems probable that previous vaginal cornification was due to development of type B cells which degenerated with age becoming filled with pigmented material or proliferated and became invasive without producing oestrogen. The female with bilateral cortical lymphoma ceased to produce oestrus cells at least one month before death. There was evidence of previous secretion in the nipple regions which had developed but were stunted. There was scattered lobular-alveolar differentiation and no nodules. Salivary glands and glomerular capsules were male in type. In 6 males, castrated at the same age, which died from 16 to 18 months of age, no prostates had developed ; salivary glands were all male ; mammary rudiments had grown out in 5 ; type B cells were present in all adrenals and were hyperplastic in 4.

Type, site and age distribution of mammary carcinomas

Of 26 induced carcinomas including 2 double primaries, 21 were in anterior pairs and 5 in posterior nipple regions (Table III).

Of 41 spontaneous tumours seen in breeders and virgin females bred in these laboratories, 31 were in anterior and 10 in posterior nipple areas. The difference between spontaneous and induced is not significant. From Table III it will be seen that the age distribution of tumour occurrence was substantially the same for the spontaneous and induced tumours. The induced morphological types were also similar with the exception that no carcino-sarcoma was seen. Eleven were of type A, 9 of type B, 2 of type C, 2 were adenoacanthomas, one included regions of A and C formations and one included A, B, and anaplastic cells according to the descriptions of Dunn (1959). No chronic inflammatory foci were found, nor were cholesterol esters, but the latter were not sought for as systematically as in the RIIIf strain (Pullinger, 1949).

Grafts

Grafts of all the induced tumours which were tested grew in C_3Hf hosts or their F_1 hybrids. Eleven out of 13 grew in males and all 19 which were grafted into females. Frozen sections stained with Sudan IV revealed lipoid material in large alveoli at the periphery and in central regions of a graft of a spontaneous mammary carcinoma which was chosen at random and had grown for 3 months in an intact male before it was examined. Numerous macrophages laden with sudanophil particles were also found.

Experiment V

Of 20 virgin females ovariectomised at 62 to 78 days of age and given 200 μg. weekly of oestriol, 18 lived to one year or over. Of 4 that have died up to the present time, 15 months old, none has developed urinary calculi and those alive show no signs of it. At 9 to 10 months old the average weight was 4 g. less than that of untreated controls of the same age. Weights have not increased since then. In the early months of experiment, uterus and vagina became distended with oestrus products but this caused no urethral obstruction. The vaginas and uteri of 3 that died after 10 months of age were distended but empty and the epithelium was atrophic. At one year and at 14 months old mammary glands of 2 mice were developed beyond the pubertal stage but were "stunted" as described by Gardner (1941). There was some scattered lobular-alveolar differentiation and several hyperplastic nodules which could not be accurately counted because the glands were not involuted.

Partial ovariectomy

One of 22 virgin females from each of which one ovary was completely and one partially excised, developed mammary carcinoma. This result was similar to that obtained in the RIIIf strain (Pullinger, 1957).

COMMENT

The induction of mammary carcinoma with oestrogens in ovariectomised mice lacking the mammary tumour agent was dependent on an inherited susceptibility

TABLE III.—*Distribution in Nipple Regions and Time of Appearance of Spontaneous and Induced Mammary Carcinoma*

Hormonal status or treatment and number of mice	Nipple areas	Age in months 12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	Total tumours Anterior	Posterior
Breeders, intact (148)	A	1	—	—	1	—	1	3	1	2	4	—	1	3	3	2	2	2	1	2	—	—	29	—
	P	—	—	—	1	—	1	1	1	—	—	2	2	—	—	—	—	—	2	—	—	—	—	10
Virgins, intact (118)	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	2	—
	P	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
																							31	10
Virgins, ovariectomy and—																								
Oestrone 5 μg. (30)	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	P	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	1
Oestrone 10 μg. (44)	A	—	—	—	—	1	1	—	—	—	1*	3	1	—	—	—	2	1	—	—	—	—	10	—
	P	—	—	—	—	—	1	—	—	—	1*	—	—	—	—	—	—	—	—	—	—	—	—	2
Oestriol 10 μg. (34)	A	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	1	—
	P	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Oestrone Oestriol Oestradiol 10 μg. (42)	A	—	—	1	—	—	—	1	—	—	2*	—	1	1	1	1	—	—	—	1	1	—	10	—
	P	—	—	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	—	—	—	—	—	2
																							21	5

A = Anterior; P = Posterior. * = 2 primaries in the same mice and both recorded.

to this response. This susceptibility is not a characteristic of all strains, the C_{57} black for example lacks it (Shimkin, 1945, Table IV). Any attempt to find a relationship between tumour incidence and the amount of oestrogen administered depends on freedom of the strain from biologically detectable mammary tumour agent, on an adrenal cortex that responds to ovariectomy without production of endogenous oestrogens or of negligible amounts and on tolerance for considerable amounts of administered oestrogens. The C_3Hf and RIIIf strains have met these requirements except the last. The amount of oestrone that could be given to RIIIf mice did not exceed 5 μg. weekly on account of greatly increased prevalence of lymphoma and leukaemia (Pullinger, 1952, 1955, 1957). Larger amounts given to C_3Hf females gave rise to urinary calculi. Perhaps owing to these limitations which may also represent natural physiological ones, the highest incidence of mammary carcinoma attained in both these strains was the same as that characteristic for normal breeders.

Two of the results in this series of experiments in virgin females and breeders indicate that the part played by oestrogens in mammary tumour induction where there is a heritable tendency to this response is not due solely to the morphogenic function of oestrogens in an organ which would not exist without them. The breeding females which were ovariectomised had suckled several litters. Their mammae can be presumed to have been developed, yet the tumour incidence was reduced to 1 in 50 and this one was associated with a secreting adrenal cortical carcinoma. When oestrogen was substituted after ovariectomy, incidence rose with increase in the dose given and was restored to that characteristic for breeders of the strain (Pullinger, 1960*b*, 1961). Secondly, the morphological responses in the mammae of ovariectomised virgin females to two different doses of oestrogen never attained the full development seen in pregnancy, yet the same high incidence as in normal breeders was reached with the higher dose. From this observation it may be concluded that tumour incidence is not related to the number of the cell population at risk but that there are differences in responsiveness among mammary cells to the oestrogenic stimulus acting in combination with pituitary hormones.

It cannot be assumed that quantitative tests of the mammary tumour inducing capacity of oestrogens would give similarly consistent results if applied to heterozygous stocks less sensitive to environmental stimuli yet including susceptible individuals. Test stocks could be bred at random from strains with known susceptibilities, free from demonstrable mammary tumour agent and preferably lacking the capacity to produce a post-castrational oestrogen or secreting it in negligible amounts. Some C_{57} black strains are free of agent, are insusceptible to oestrogen-induced tumours and do not secrete post-castrational oestrogen. The RIIIf and C_3Hf are susceptible and meet the other requirements. Both the DBAf (Mühlbock, van Ebbenhorst Tenbergen and van Rijssel, 1953) and the O_{20}f (Mühlbock and van Rijssel, 1955) are susceptible and have been freed of agent but the DBAf secretes post-castrational oestrogen (Woolley, Fekete and Little, 1939). No data in this respect were found for the O_{20}, in published papers.

SUMMARY

1. Mammary carcinoma was increased from 2 per cent in intact C_3Hf virgin female mice to 25 and 26 per cent respectively in those ovariectomised and given

10 μg. weekly of oestrone alone or of a mixture of oestrone, oestriol and oestradiol for 60 weeks. Adenoma increased from 29 to 95 and 71 per cent respectively. The carcinoma incidences equalled that for intact breeding females of the strain.

2. One mammary carcinoma occurred among 34 ovariectomised virgin females given 10 μg. weekly of oestriol for 60 weeks. These mice were free of urinary calculi as were 20 similarly treated with 200 μg. weekly and observed for 15 months.

3. Microscopic invasive adrenal cortical carcinoma occurred in some old ovariectomised females after the end of treatment. With this exception and possibly lymphomas, tumours did not occur more frequently in remaining sites than in untreated mice.

REFERENCES

Boot, L. M. and Mühlbock, O.—(1956) *Acta Un. int. Cancr.*, **12**, 569.

Dunn, T. B.—(1959) in ' Physiopathology of Cancer ', edited by Homburger, F. and Fishman, W. H. London (Cassel & Co. Ltd.), 2nd edition, p. 38.

Foulds, L.—(1956) *J. nat. Cancer Inst.*, **17**, 783.

Gardner, W. U.—(1941) *Endocrinology*, **28**, 53.

Heston, W. E., Deringer, M. K. and Vlahakis, G.—(1960) *J. nat. Cancer Inst.*, **24**, 721.

Lipschutz, A.—(1925) *Brit. J. exp. Biol.*, **2**, 331.

Mühlbock, O., van Ebbenhorst Tenbergen, W. and van Rijssel, Th. G.—(1953) *J. nat. Cancer Inst.*, **13**, 505.

Idem and van Rijssel, Th. G.—(1955) *Ibid.*, **15**, 73.

Pullinger, B. D.—(1949) *Brit. J. Cancer*, **3**, 494.—(1952) *Ibid.*, **6**, 69.—(1955) *Ibid.*, **9**, 613.—(1957) *Ibid.*, **11**, 249.—(1959) *Ibid.*, **13**, 99.—(1960*a*) *Ibid.*, **14**, 279.—(1960*b*) *Ibid.*, **14**, 502.—(1961) *Ibid.*, **15**, 127.

Idem and Iversen, S.—(1960) *Ibid.*, **14**, 267.

Schenken, J. R. and Burns, E. L.—(1943) *Cancer Res.*, **3**, 693.

Iidem and McCord, W. M.—(1942) *Endocrinology*, **30**, 344.

Shimkin, M. B.—(1945) in a ' Symposium on Mammary Tumors in Mice '. Washington (Amer. Ass. Advanc. Sci.), p. 85.

Woolley, G. and Little, C. C.—(1945) *Cancer Res.*, **5**, 193.

Idem, Fekete, E. and Little, C. C.—(1939) *Proc. nat. Acad. Sci., Wash.*, **25**, 277.

ANTIGENIC COMPOSITION OF NORMAL TISSUES AND TUMOURS IN AN INBRED STRAIN OF MICE. CHANGES IN CERTAIN GLOBULINS ASSOCIATED WITH TUMOUR GROWTH

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PREVIOUS work (Pikovski and Witz, 1961) on soluble antigens of normal tissues and mammary tumours from mice of the RIII strain showed the presence of specific antigens of protein nature in the liver and kidney. There was also an indication—though no definite proof—that a specific antigen of mammary glands was lacking in mammary tumours.

It was also shown that in the transplantable and spontaneous mammary tumours of the RIII strain, a soluble antigen of protein nature was present in a much higher concentration than in the normal tissues of mice of this strain.

The work of other investigators, employing various serological methods in dealing with different cell components and protein fractions, also indicated quantitative and qualitative differences in the antigens of tumours as compared to the corresponding normal tissues of humans and animals.

Most of this work has been well reviewed (Hauschka, 1952; Zilber, 1958; Southam, 1960); we shall refer mainly to studies on soluble antigens.

The loss of unsoluble as well as partly soluble specific tissue antigens in tumours originating in these tissues was demonstrated by different serological methods (Weiler, 1956; Nairn *et al.*, 1960).

Increase as well as decrease in some soluble antigens of mouse tumours were demonstrated by Easty and Ambrose (1957), Abramoff *et al.*, (1959), Plescia *et al.*, (1959) and Levina (1959).

Similar changes were also reported in soluble antigens of human cancers by Witebsky *et al.* (1956), Gorodilova and Shershulskaia (1959) and Zilber (1958); and in human leukemic cells by Seligmann, Grabar and Bernard (1959).

The first part of the present study deals with a specific antigen of the mammary glands lacking in mammary tumours, the second with the evaluation of the presence of an antigen in a transplantable mammary carcinoma and in normal tissues, serum and urine of tumour-bearing mice of the same strain.

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MATERIALS AND METHODS

The mice in these experiments belonged to the RIII strain (Syn : Paris R3). The subline used was inbred by us for 30 generations. The strain was highly cancerous for at least 16 generations, but then the cancer incidence gradually began to decrease, being very low at present. The strain of the transplantable mammary carcinoma(MMClA) described previously (Pikovski and Schlesinger, 1955 and 1959) started from a spontaneous tumour which occurred in the 8th generation, at the time when the strain showed a high mammary tumour incidence, and was since transplanted exclusively in this strain. The tumours used, were from the 100–150th passages.

Tissue extracts

Freshly excised normal organs, or carefully selected non-haemorrhagic parts of tumours were finely cut up, distilled water was added, the mash lyophylized and kept in closed containers over silica gel at 4° C. For the preparation of extracts, the dry tissues were powdered, saline added to necessary concentration: the suspension was then homogenized and centrifuged at 18,000 × g. The supernate was distributed 1–5 c.c. in test tubes, kept at −20° C. and defrosted shortly before use either for immunization, or for precipitation tests. The concentration of the tissue extracts compared on one plate was brought to a similar protein content as estimated by the Biuret- or Micro Kjeldahl method. The protein content of dry tumour and of the mixture of normal tissues was shown to be approximately equal, and amounted to half of their weight ; whereas the protein content of the dry mammary gland was 0·25 of its weight.

The highly concentrated extracts used in some experiments were prepared by lyophylizing the measured amounts of a 40 mg./ml. extract and redissolving the dry powder in a necessary amount of water.

The colostrum was obtained from the stomachs of one day old mice, homogenized with saline, centrifuged, the fat taken off and the supernate used.

The urine was collected for 17–18 hours from each mouse separately, with addition of streptomycin and penicillin.

The antisera were produced in rabbits. Suitable antisera against MMClA were obtained by 3 intramuscular injections, twice weekly, of 2 ml. tissue extract in Freund's adjuvant. This was followed a week later by an intraperitoneal booster injection of 5 ml. of extract. Ten days after the booster, 2–3 ml. of blood were obtained from the ear for a test and if the serum was found satisfactory, the rabbit was bled from the heart 4–6 days later.

However, this method proved unsuitable for the demonstration of the specific mammary gland antigen. For this purpose good antisera were obtained by 2–3 intradermal injections into the foot pads of 0·2 c.c. of a mammary gland extract (40 mg./ml.) in Freund's adjuvant.

Precipitation tests

The double diffusion gel precipitation method of Ouchterlony was used for demonstration of antigens.

The method of Björklund (1952) was used for absorption with soluble antigens by incorporating tissue extracts of various concentrations into the agar.

UMI

Tests on the nature of the antigen

(1) Commercial preparations of enzymes were incubated with the tissue extracts in a 1 : 1 ratio, as follows :

Enzyme	Concentration	pH	Incubation	
			Duration (hr.)	Temperature °C.
Trypsin	10 mg./ml.	7·6	20	37
Papain	10 mg./ml.	7·0	20	37
Ribonuclease (protease free)	750 μg./ml.	7·2	1	37
Hyaluronidase	2 mg./ml.	7·0	4	37 (with shaking)

(2) Periodate ($NaIO_4$) : 0·04M solution was mixed with the tumour extract in a 1 : 1 ratio and kept at pH 7, at room temperature for 1 hour.

(3) Precipitation of proteins with Rivanol (6·9 diamino 2 ethoxyacridine lactate) was carried out according to Saifer and Lipkin (1959). The supernate was used.

RESULTS

I. *Antigens of mammary glands*

Since the epithelium of mammary glands is only a part of the tissue taken for immunization, it was likely that a specific antigen—if present in our extracts—was in low concentration, moreover it could be also a weak antigen. Methods to obtain antibodies suitable for demonstration of this antigen were sought. It was shown that antisera obtained by intradermal injections into the foot pads of small amounts of mammary glands extract in adjuvant, revealed in the mammary glands 2–3 antigens common to a mixture of normal tissues (liver, kidney and spleen), and one antigen not shared with these tissues. This antigen produced a very straight, sharp, conspicuous band (Fig. 1). This band alone was still apparent after absorption with the extracts of mixed normal kidney, liver and spleen (Fig. 2). It was also shown that extracts of normal tissues tested in concentrations of 20–160 mg./ml. against the same antiserum, gave 2–3 bands common with those produced by mammary glands, however the straight band was never apparent.

It was further shown that extracts of mammary tumours, equally tested in concentrations from 20–160 mg./ml. against the same antiserum, gave at least 4 bands, but the straight, conspicuous band of the mammary glands was not produced by these extracts either (Fig. 3).

The absorption of the anti-mammary gland serum with tumour extracts did not remove the antibodies against the antigen producing the conspicuous straight line (Fig. 4).

Since the mammary glands were removed from pregnant mice at about 10–15 days of pregnancy, there was a possibility that the conspicuous line may have been due to an antigen of the colostrum. However, it could be demonstrated that, although the colostrum gave 4–5 bands with the antiserum against the mammary glands, the conspicuous band of the mammary gland was not produced by the colostrum.

Thus a tissue specific antigen was demonstrated in the mammary glands, this antigen was lacking in the mammary tumours. Preliminary tests on its

nature showed that it was destroyed by boiling and proteolytic enzymes, and that after the precipitation of proteins by Rivanol it remained in the supernatant fraction. It is therefore a protein, which behaves like a serum globulin.

II. *Antigens of mammary tumours*

Tests with antisera against MMClA, obtained from several rabbits, revealed that 4–6 bands were produced by extracts of MMClA. One band was of special interest as it always had a characteristic density, shape and position, and was invariably produced by every individual tumour sample of the MMClA, or by pools derived from different passages of this tumour (Fig. 5, 9, 12). Extracts of spontaneous mammary tumours of the RIII and C3H strain equally produced this conspicuous band.

After absorption with an extract of 20 mg./ml. of mixed normal tissues (kidney, liver, spleen and mammary glands) 2 bands were still obtained with tumour extracts. Absorption with extracts of 40–80 mg./ml. removed antibodies against all antigens, except one which produced the strong band, slightly curved to the serum well (Fig. 6). Absorption by each of these antigens separately did also not affect the appearance of this sharp conspicuous band.

Preliminary tests on the nature of the antigen producing the conspicuous band showed that, while it was not affected by either hyaluronidase, ribonuclease or sodium periodate, it was destroyed by boiling and by proteolytic enzymes (papain, trypsin). After precipitation of proteins by Rivanol it remained in the supernatant. Therefore, similarly to the mammary glands antigen, this was a protein behaving like a serum globulin. Although the band was not produced by extracts of normal tissues in concentrations equal to those of tumours, it was, however, not specific to tumour tissue. The use of extracts of normal tissues in a concentration of 160 mg./ml. revealed a band identical with that produced by 5 mg./ml. extracts of tumours, though situated nearer to the antigen well, indicating roughly that in normal tissues this antigen was also present but in a concentration at least 30 times lower than in the tumour. This approximate proportion was confirmed by further experiments with serial dilutions of tumour and normal tissue extracts. Undiluted antiserum was used against twofold dilutions of tissue extracts. These were all compared with an extract of MMClA at 10 mg./ml.

The use of this technique demonstrated that a visible band was still produced by extracts of MMClA in a concentration of 0·6 mg./ml. (300 μg. protein) (Fig. 9) as compared to 20 mg./ml., the lowest concentration, where a similar band was produced by extracts of mixed normal tissues. Titration of each normal organ separately showed that the band was still produced by 20 mg./ml. extracts of liver, kidney, mammary glands and lymph glands, though not less than 80 mg./ml. were required to obtain a band by extracts of spleen.

A different picture was observed when the amount of the MMClA antigen in normal organs of tumour-bearing mice was investigated, this was shown to be considerably higher than in the organs of normal mice. At the concentration of 5 mg./ml., a visible band was still produced by the extracts of mixed normal tissues, or by separate extracts of kidney, liver, and lymph glands from tumour-bearing mice, as compared to 20 mg./ml. of corresponding tissues from normal mice. However, the spleen extracts from tumour-bearing mice produced a visible band only in concentrations beginning from 20 mg./ml., as compared to

80 mg./ml. of normal spleen extracts (Table I). It was thus indicated that the concentration of this antigen is approximately 4 times higher in each of the organs of tumour-bearing mice as compared with the corresponding organs of normal animals.

TABLE I.—*Lowest Concentration of Extracts of Lyophylized Tissues (mg./ml.) where the MMC1A Band was still Visible*

	MMC1A	Liver	Kidney	Mammary glands	Spleen	Lymph glands
Normal mice	—	20	20	20	80	20
Tumour-bearing mice	0·6	5	5	5	20	5

This proportion was found to be similar in sera. In pools of sera from normal mice and those bearing mammary tumours (MMC1A) of different passages, an antigen was demonstrated producing a band identical with the conspicuous band of extracts of MMC1A. The concentration of this antigen in sera of tumour-bearing mice was, however, 4–8 times higher than in sera of normal mice.

The different nature of the mammary gland antigen and the mammary tumour antigen was revealed by the following experiment. The combination of wells as designed by Abelev (1960) was used to compare antigens of two antigen-antibody systems, where common antigens would produce parallel bands, while bands produced by different antigens would cross. Comparing the bands produced by the mammary glands and by the mammary tumours with their corresponding

EXPLANATION OF PLATES

FIG. 1.—A. Anti-mammary gland serum. B. Mammary gland. C. Mixture of liver, kidney and spleen.

FIG. 2.—A, B, C. Same as Fig. 1. Absorption with mixture of liver, kidney and spleen.

FIG. 3.—A. Anti-mammary gland serum. B. Mammary gland. C. Mammary tumour (MMC1A).

FIG. 4.—A, B, C. Same as in Fig. 3. Absorption with mammary tumour (MMC1A).

FIG. 5.—A. Anti-mammary tumour (MMC1A) serum. B. MMC1A. C. Mixture of liver, kidney, spleen and mammary gland.

FIG. 6.—A, B, C. Same as in Fig. 5. Absorption with mixture of liver, kidney, spleen and mammary gland.

FIG. 7.—A. Mammary tumour (MMC1A). B. Mammary gland. C. Anti-mammary tumour (MMC1A) serum. D. Anti-mammary gland serum.

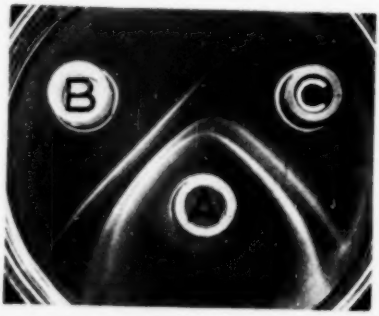
FIG. 8.—A. Mammary gland. B. Mammary tumour (MMC1A). C. Anti-mammary gland serum. D. Anti-mammary tumour (MMC1A) serum.

FIG. 9.—A. Anti-mammary tumour (MMC1A) serum. B. MMC1A (10 mg./ml.). C. MMC1A (5 mg./ml.). D. MMC1A (2·5 mg./ml.). E. MMC1A (1·25 mg./ml.). F. MMC1A (0·62 mg./ml.). G. MMC1A (0·31 mg./ml.).

FIG. 10.—A. Anti-mammary tumour (MMC1A) serum. B. MMC1A (10 mg./ml.). C. Urine of a male RIII mouse, bearing MMC1A 18 days after grafting, tumour size 3·2 × 2·2 cm. D. Urine of the same mouse as in C, 12 days after grafting, tumour size 2·7 × 1·6 cm. E. Urine of a normal male RIII mouse.

FIG. 11.—A. Anti-mammary tumour (MMC1A) serum. B. MMC1A (10 mg./ml.). C. Urine of a male RIII mouse, bearing MMC1A, 16 days after grafting, tumour size 3·6 × 2·6 cm. D. Urine of a female RIII mouse, bearing MMC1A, 16 days after grafting, tumour size 3·2 × 2·4 cm. E. Urine of the same mouse as in C, 20 days after grafting, tumour size 3·9 × 2·8 cm. F. Urine of the same mouse as in D, 20 days after grafting tumour size 3·5 × 2·5 cm. G. Urine of a normal female RIII mouse.

FIG. 12.—A. Anti-mammary tumour (MMC1A) serum. B. MMC1A (10 mg./ml.). C. Urine of a female C3H mouse bearing a spontaneous mammary tumour, size 1·3 × 1·2 cm. D. Urine of a female C3H mouse bearing spontaneous mammary tumour, size 2·3 × 1·8 cm. E. Urine of a normal female C3H mouse. F. Empty.



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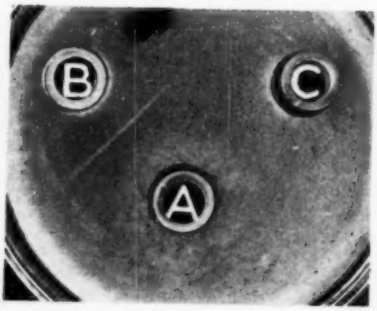

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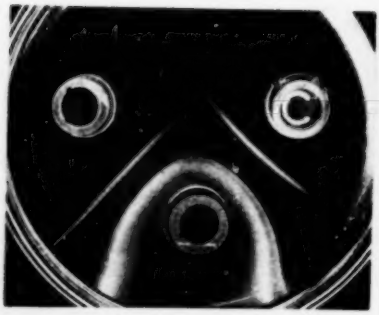

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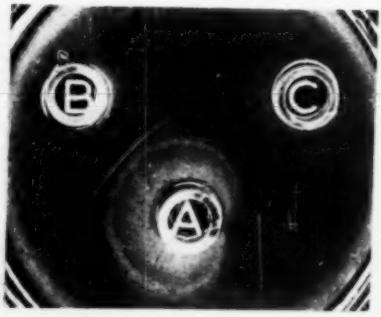

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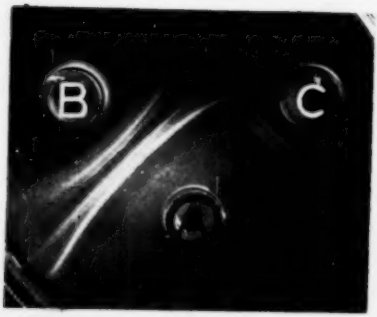

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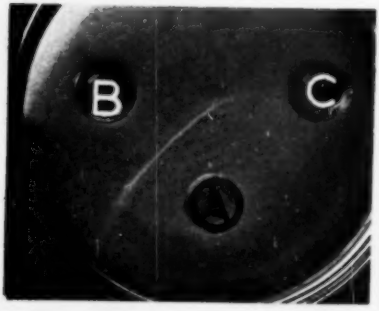

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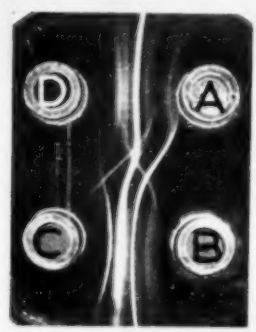

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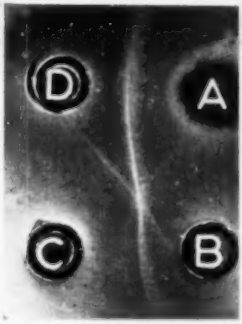

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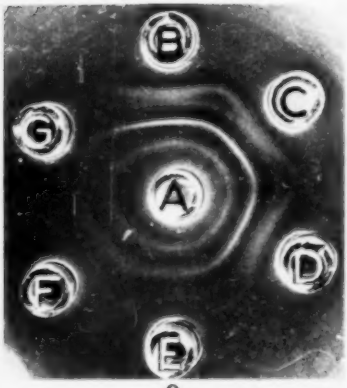

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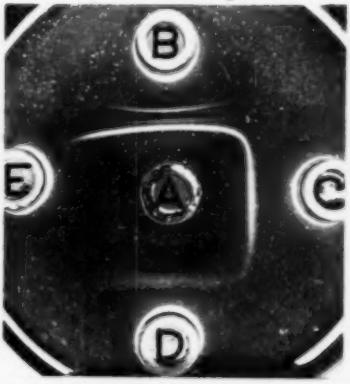

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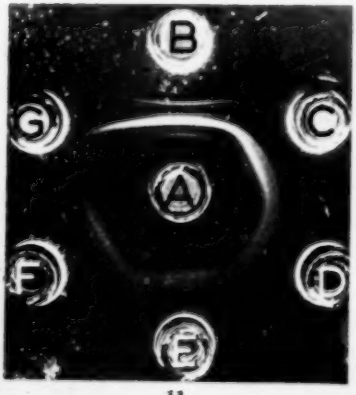

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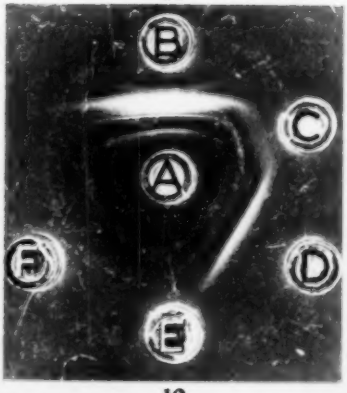

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antisera, we observed that while three bands were produced by common antigens, two or more bands definitely crossed (Fig. 7). The absorption with normal tissue antigens by the addition of their extracts to the dish resulted in the production of two distinctly crossed bands and one straight band Thus two different antigens were demonstrated in mammary tumours and mammary glands, both absent in other normal tissues in equal concentrations, and also one antigen common to mammary glands and mammary tumours, but not present in other normal tissues (Fig. 8).

It is interesting to note that the first two bands of low density, seen next to the antigen well (Fig. 9) were also produced by tumour extracts but *not* by normal tissue extracts when used at concentrations of 10–20 mg./ml. The position of these bands was also followed up in the titration experiments and it could be shown that while the 2·5 mg./ml. extracts of mammary tumours could produce these 2 bands, not less than 40 mg./ml. extracts of liver or kidney from normal mice were required to produce similar bands. These antigens could not be detected in extracts of spleen and lymph glands even at such high concentrations as 40–80 mg./ml.

Apparently, though the actual concentrations of these antigens in tumours and in normal tissues are lower than those of the antigen producing the conspicuous band, their concentration in tumour is still approximately 16 times higher than in the normal tissues. Both antigens were destroyed by trypsin and by boiling, and appeared in the soluble fraction after treatment with Rivanol.

Globulin in urines of tumour-bearing mice

The investigation on the presence of the antigen producing the strong MMClA band, in the urines of mice bearing the transplantable MMClA mammary carcinoma, was started 8 days after grafting and continued up to the death of the mouse. The urines were collected every 4–6 days from the same individual mice, or—in some experiments—pooled from 2–4 mice with tumours of the same passage. In the latter case, the urines of males and females were pooled separately. The tumours were measured before collecting the urines. Urines of normal male and female mice were collected at the same time and tested in the same petri dishes. The antigens of the urines were tested against anti-MMClA sera and compared with those of the MMClA extracts.

The purpose of some experiments was to compare urines of males and females taken at the same period ; in others the urines of either males or females collected at different periods, were compared.

Urines of mice with spontaneous RIII or C3H tumours of various sizes were also investigated and compared with the urines of normal mice from the corresponding strain.

It was shown that urine taken 8 days after grafting, did not produce a visible MMClA band, but 12 days after grafting a clear band appeared, identical with the MMClA band, and 4–6 days later, when the average tumour size reached 3·4 × 2·5 cm., a rather strong band was invariably observed (Fig. 10, 11). In urines obtained after this period the density of the band did not increase and in some cases was even reduced. There was no definite difference in the density and position of this urinary band in males and females, though individual differences were sometimes observed.

The urines of mice with spontaneous mammary tumours produced in some cases a weak band when the tumour was scarcely palpable, while a strong band was invariably observed when tumours approached the average size of 2·3 × 1·8 cm. (Fig. 12). When the tumour grew further, the density of the line produced by urine did not change for at least 3 weeks and then grew weaker though it was still clear, up to the death of the animal. It should be noted, however, that at this stage, though the tumours were actually large, their size was accounted for by large necrotic masses and interstitial fluid.

No trace of the MMClA band was ever observed when urines of normal C3H mice were tested. Tests with normal RIII mice indicated in 3 out of 20 urines a weak band appearing in the same plates, where the urines of tumour bearing mice produced strong bands.

It is interesting to note that besides the characteristic tumour band, another weaker band, nearer to the serum well, was produced by the urines of tumour-bearing and of normal mice. This band was also obtained from the extracts of tumours as well as of normal tissues.

The antigens producing both urinary bands were destroyed by boiling and remained in the soluble fraction after treatment with Rivanol, indicating that two globulins were excreted in mouse urine, one of them predominantly by tumour-bearing and the other also by normal mice.

A further test of mammary tumour extracts against the anti-tumour and the anti-urine sera carried out simultaneously, revealed that 2 identical bands appeared with both antisera. Thus the tumour and the urine of tumour-bearing mice elicited two immunologically identical antibodies.

DISCUSSION

The first part of this study indicates the presence of an organ specific antigen in the mammary glands, which is lacking in the mammary tumours. This finding is similar to those of Weiler (1956) and Nairn *et al.* (1960). However, it seems yet premature to draw any conclusions as to its relation to the process of carcinogenesis.

The second part shows a considerable increase of an antigen in the mammary tumours as compared to the homologous and other normal tissues. Both antigens behaved like serum globulins, but were shown to be immunologically different.

More will be known on the position of these antigens among the globulin fractions and on their similarity with serum globulins, after the completion of immunoelectrophoretic studies now in progress. However, the work of Korngold and van Leeuven (1957) with purified serum proteins permits their tentative identification by the Ouchterlony method. It was shown that the gamma-globulin produced a completely straight band, such as was obtained from the specific mammary glands antigen (Fig. 1, 3); whereas antigens with smaller molecular weight produced bands curved to the serum container, such as produced by the tumour antigen (Fig. 5, 9). Furthermore, Korngold has also shown that the gamma-globulins are weaker antigens than the alpha- and beta-globulins which could be analogous with the characteristics of the weak mammary gland antigen and the strong tumour antigen. The latter produced an identity band with that of an antigen shown to increase 4–8 times in serum of tumour-bearing

animals. The same antigen was also shown to be present in organs of tumour-bearing mice in a concentration 4 times higher than in the organs of normal mice. The increase of this and another two, weaker antigens in the organs of tumour-bearing animals cannot be explained by hypervolaemia, since the blood volume of liver and kidney is known to increase to only about 30 per cent, while a decrease occurs in the blood space of spleen of tumour-bearing, as compared to normal mice (Greenstein, 1954). This increase in liver and kidney can rather be explained by the higher amount of the antigen in the serum, while an increase in the lymph glands is more plausibly due to the increase of the antigen in the lymph, though it may also be due to an entirely different mechanism.

An immunologically identical globulin fraction was also found to be excreted in urines of tumour-bearing mice.

Through quantitative and qualitative changes in the soluble antigens of malignant tissues were repeatedly reported, little information is available (Mann and Welker, 1940) on the possible similarity of changes in tissue proteins and those known to occur in the spectrum of serum proteins of cancer patients and tumour-bearing animals.

The decrease in albumins, as well as quantitative and qualitative changes in the serum globulin fractions were repeatedly revealed by Ouchterlony gel precipitation and immunoelectrophoresis. Thus, Darcy (1957, 1960) demonstrated that an alpha-globulin increased in plasma of rats with transplantable and induced tumours, as well as in sera of pregnant rats and those with regenerating liver ; the increase being apparently due to growth processes. Bernfeld *et al.* have found an abnormal alpha globulin and quantitative changes in other alpha and gamma globulin fractions in sera of mice with transplantable (Bernfeld and Homburger, 1955) and spontaneous tumours (Miller and Bernfeld, 1960) as well as similar alterations in sera of patients with lung carcinoma (Bernfeld and Miller, 1960). Clausen *et al.* (1959, 1960) and Rask-Nielsen, Gormsen and Clausen (1959) demonstrated a marked increase of the beta 2 III fraction in sera of mice with leukaemia and with spontaneous or transplantable tumours, while an increase in the beta 2 I fraction and decrease of gamma globulin was observed in sera of mice with hepatoma. The increase of the beta fraction was explained as an immunological response to tumour proteins.—Reviewing numerous data on tumour antigens, Zilber (1958) suggested that " carcinogenesis involves first of all a disturbance of globulin synthesis ".

The present study revealed a similarity in the behaviour of tumour and serum globulins, since a protein found in high amounts in tumours produced an identity band with an antigen which was found also in increased amounts in sera, and which also appeared in urines of tumour-bearing mice. This protein was probably not related to that found by Darcy, since it was not present in embryos and did not increase in sera of pregnant mice. It is more likely to resemble the increasing serum globulin fractions, described by Rask-Nielsen *et al.* (1959).

At the present stage of research, however, there is no convincing evidence on the role and origin of this globulin. It may be produced and released by the tumour cells, or it may occur in abnormal amounts at another location, as a result of a disturbance in globulin synthesis, caused by tumour-growth, and subsequently be selectively absorbed by the tumour. It may possibly be an immunological response to tumour antigens.

It is also not known whether there is a causative relationship between the

disappearance of a specific organ antigen and the increase of another protein in the tumour of this organ, or are these only two parallel processes.

The antigen may be similar to that found in RIII mice by Plescia *et al.* (1959), but the possibility of its viral origin has still to be investigated.

These speculations all need experimental evidence and can only indicate ways for further research.

SUMMARY

A specific tissue antigen of globulin nature was demonstrated in the mammary glands of the RIII mice. This antigen was lacking in the mammary tumours of mice of the same strain.

An immunologically different antigen of globulin nature was demonstrated in spontaneous and transplanted mammary tumours of mice of the same strain in a concentration at least 30 times higher than in several tissues of normal mice.

This antigen was found in normal tissues and serum of tumour-bearing animals in a concentration 4 times higher than in the corresponding tissues of normal mice.

An immunologically identical globulin was shown to be present in considerable amounts in the urines of mice bearing spontaneous and transplantable mammary tumours.

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REFERENCES

Abelev, G. I.—(1960) *Fol. Biol.*, **6**, 56.

Abramoff, P., Chinchinian, H. and Saunders, J. W.—(1959) *J. nat. Cancer Inst.* **22**, 919.

Bernfeld, P. and Homburger, F.—(1955) *Cancer Res.*, **15**, 359.

Idem and Miller, E. E.—(1960) *Proc. Amer. Ass. Cancer Res.*, **3**, 95.

Björklund, B.—(1952) *Proc. Soc. exp. Biol. N.Y.*, **79**, 319.

Clausen, J., Heremans, J., Heremans, M. Th. and Rask-Nielsen, R.—(1959) *J. nat. Cancer Inst.*, **22**, 57.

Idem, Rask-Nielsen, R., Christensen, H. E. and Munkner, T.—(1960) *Cancer Res.*, **20**, 178.

Darcy, D. A.—(1957) *Brit. J. Cancer*, **11**, 137.—(1960) *Ibid.*, **14**, 524, 534.

Easty, G. C. and Ambrose, E. J.—(1957) *Ibid.*, **11**, 287.

Gorodilova, V. V. and Shershulskaia, L. V.—(1959) 'Pathogenesis and Immunology of Tumours'. London (Pergamon Press Ltd.), p. 101.

Greenstein, J. P.—(1954) 'Biochemistry of Cancer'. Second edition. New York (Academic Press Inc.), p. 507.

Hauschka, T. S.—(1952) *Cancer Res.*, **12**, 615.

Korngold, L. and Van Leeuven, G.—(1957) *J. Immunol.*, **78**, 172.

Levina, D. M.—(1959) 'Pathogenesis and Immunology of Tumours'. London (Pergamon Press Ltd.), p. 113.

Mann, L. S. and Welker, W. H.—(1940) *Amer. J. Cancer*, **39**, 360.

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MILLER, E. E. AND BERNFELD, P.—(1960) *Cancer Res.*, **20**, 1149.
NAIRN, R. C., RICHMOND, H. G., MCENTEGART, M. G. AND FOTHERGILL, J. E.—(1960) *Brit. med. J.*, ii, 1335.
PIKOVSKI, M. AND SCHLESINGER, M.—(1955) *Cancer Res.*, **15**, 285.—(1959) *Ibid.*, **19**, 222.
Idem AND WITZ, I.—(1961) *Acta Un. int. Cancr.*, **17**, 244.
PLESCIA, O. J., DOAT, P., PONTIERI, G. AND BIANCO, A.—(1959) *Fed. Proc.*, **18**, Abs. No. 2325.
RASK-NIELSEN, R., GORMSEN, H. AND CLAUSEN, J.—(1959) *J. nat. Cancer Inst.* **22**, 509.
SAIFER, A. AND LIPKIN, L. E.—(1959) *Proc. Soc. exp. Biol. N.Y.*, **102**, 220.
SELIGMANN, M., GRABAR, P. AND BERNARD, J.—(1955) *Sang*, **26**, 52.
SOUTHAM, C. M.—(1960) *Cancer Res.*, **20**, 271.
WEILER, E.—(1956) *Z. Naturf.*, **11**, 31.—(1956) *Brit. J. Cancer*, **10**, 553.—(1956) *Ibid.*, **10**, 560.
WITEBSKY, E., ROSE NOEL, R. AND SHULMAN, S.—(1956) *Cancer Res.*, **16**, 831.
ZILBER, L. A.—(1958) *Advanc. Cancer Res.*, **5**, 291.

THE TOXIC EFFECTS OF IRON-DEXTRAN COMPLEX ON MAMMALIAN CELLS IN TISSUE CULTURE

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RECENT work (Richmond, 1957, 1959, 1960) has shown that iron-dextran complex is carcinogenic in the rat and mouse. These observations have been confirmed and extended by Haddow and Horning (1960). The dextran component of the complex is inactive, and whether the iron alone or the metal complex is the carcinogenic agent, the arresting problem of mechanism of action remains to be solved. While this question is of fundamental interest, there is a direct bearing on some aspects of human cancer since an association between lung cancer and metal in man has become clear in recent years. Kennaway and Kennaway reported in 1936 that lung cancer in England was over twice as common in metal grinders as in the general population. Haematite miners (Faulds and Stewart, 1956) and asbestos workers (Bonser, Faulds and Stewart, 1955) are also particularly prone to develop lung cancer. The similar occurrence of carcinoma of the lung in haematite miners, asbestos workers and chromate workers has been stressed by Faulds (1957) and since both asbestos and chromate contain varying proportions of iron in their chemical composition, it is possible that iron is the common carcinogenic factor. Attention may also be drawn to the high incidence of hepatoma in haemochromatosis (Warren and Drake, 1951) and the occurrence of carcinoma in relation to foreign metallic bodies (McDougall, 1956; Siddons and MacArthur, 1952).

With these facts in mind, it was considered desirable to investigate the effects of iron-dextran on cells grown *in vitro*, a system which allows fewer experimental variables than the intact animal. The present study concerns the acute reactions of growing cells *in vitro* on a short term basis. From previous experience *in vivo*, it was expected that a relatively high dose of iron-dextran would be necessary to produce immediate visible toxic damage, and this proved to be correct. It was hoped that elucidation of the mechanism of the immediate toxic reaction would afford some insight into the possible carcinogenic mechanism.

MATERIALS

The several cell strains used in this investigation included human lung, kitten lung, Chang conjunctiva, Fernandez amnion and McCoy fibroblast. Cytological changes were observed by phase contrast microscopy in the living state and, after fixation, by bright field microscopy using preparations stained by May-Grünwald-Giemsa, Feulgen's reaction, crystal violet and Perl's method for iron.

Cultures were treated for varying periods of time by different concentrations of iron-dextran complex (Benger). This preparation consists of iron complexed

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with a low molecular weight dextran fraction ; each 5 ml. contains the equivalent of 250 mg. iron calculated as Fe and present in the form of ferric hydroxide. The appropriate volume of iron-dextran was incorporated in the basic Eagle's medium with 10 per cent horse serum. Control cultures received simultaneous changes of normal medium ; in some experiments the control cultures were treated with normal medium containing a volume of sterile normal saline corresponding to the dose of iron-dextran.

In cell population studies, the Coulter electronic cell counter was used. The most suitable calibrations for counting were found to be a threshold of 20 and an aperture current setting of 4. As the error of the instrument count is in the range of 1 to 2 per cent, it did not appear necessary to correct for this error.

EXPERIMENTAL

Effect of iron-dextran on growth of cell strains

Equal numbers of cells, in the region of 200,000, were suspended in 5 ml. medium and implanted in T-30 flasks. After a period of 24 hours, during which the cells became firmly attached to the glass, the cells from 5 random flasks were harvested and counted in order to ensure that there had been equal distribution of cells to each flask. The remaining flasks were treated in groups of 5 with varying concentrations of iron-dextran except for a group of controls which received a change of normal medium. After a further 4 or 5 days at 37° C., the cells were harvested by trypsinization (1 per cent trypsin—Difco) for 3 minutes and their number was estimated, after suitable dilution in filtered saline, using the Coulter cell counter.

All strains showed a similar response to varying concentrations of iron-dextran, and the results in a kitten lung cell strain are depicted in Fig. 1 where each total represents the average content of 5 flasks. It will be seen that at a concentration of 0·5 per cent iron-dextran there is certainly no inhibition of growth, and there appears to be a slight stimulation. With increasing concentration of iron-dextran, growth is progressively inhibited and at the end of 4 days *in vitro* treatment, less than half the original number of cells implanted are now present in the flasks treated with 10 per cent iron-dextran. At concentrations of iron-dextran of 20 per cent and above, the cells were killed and " frozen " on the glass surface of the bottles.

Daily counts of population show a nearly logarithmic increase in the cell number in untreated control cultures, as seen in Fig. 2, where each point on the graph represents the average cell content of five T-30 flasks. Using 5 per cent iron-dextran in the medium, there is some inhibition of cell growth at 24 hours : after 48 hours the growth curve flattens and settles out into a plateau at a level which represents about one-half the potential growth in control cultures. The presence of 10 per cent iron-dextran in the medium leads to an immediate and continued fall in cell population.

Antagonistic effect of cobalt on toxic action of iron-dextran

It was found that if cells were maintained continuously in a medium containing iron-dextran and cobalt, the cell numbers fell rapidly owing to the toxic action of cobalt. However, a protective effect due to the presence of cobalt could be demonstrated by the following technique.

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Equal numbers of cells were implanted in T-30 flasks and, after 24 hours, groups of 5 flasks were treated for 6 hours by iron-dextran with and without cobaltous sulphate. Thereafter, the cultures were washed twice in Gey's balanced salt solution and fed with normal medium. Control cultures received corresponding changes of medium and cell populations of all flasks were estimated by the Coulter

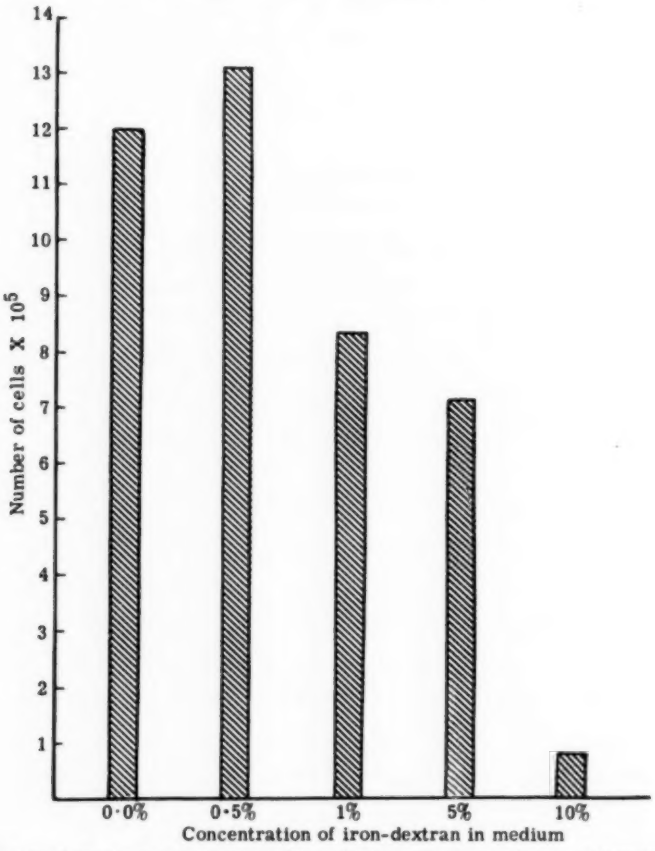


FIG. 1.—Effect of varying concentrations of iron-dextran on cell populations (kitten lung strain) after 4 days growth in treated medium.

counter after 3 days growth following initial iron-dextran treatment. Under these conditions, the protective influence of cobalt became clear in the cells treated with 10 per cent iron-dextran (Fig. 3). Six hours' exposure to 10 per cent iron-dextran was moderately toxic to the cell cultures, but in the presence of iron-dextran and $M/10^6$ cobaltous sulphate, the toxic effect of iron was obviated and a mild stimulation of cell growth became evident. A combination of 10 per cent iron-dextran and $M/10^5$ cobaltous sulphate gave a reading similar to 10 per cent iron-

dextran alone, presumably due to cobalt toxicity, and this poisonous effect was more pronounced in the cultures treated with iron and M/10^4 cobaltous sulphate.

In experiments carried out with 2·5 per cent iron-dextran in the medium, 6 hours treatment notably stimulated cell growth (Fig. 4) and the simultaneous presence of M/10^6 cobaltous sulphate had no effect on this growth increase. Increasing the concentration of cobalt to M/10^5 during the 6 hour treatment period

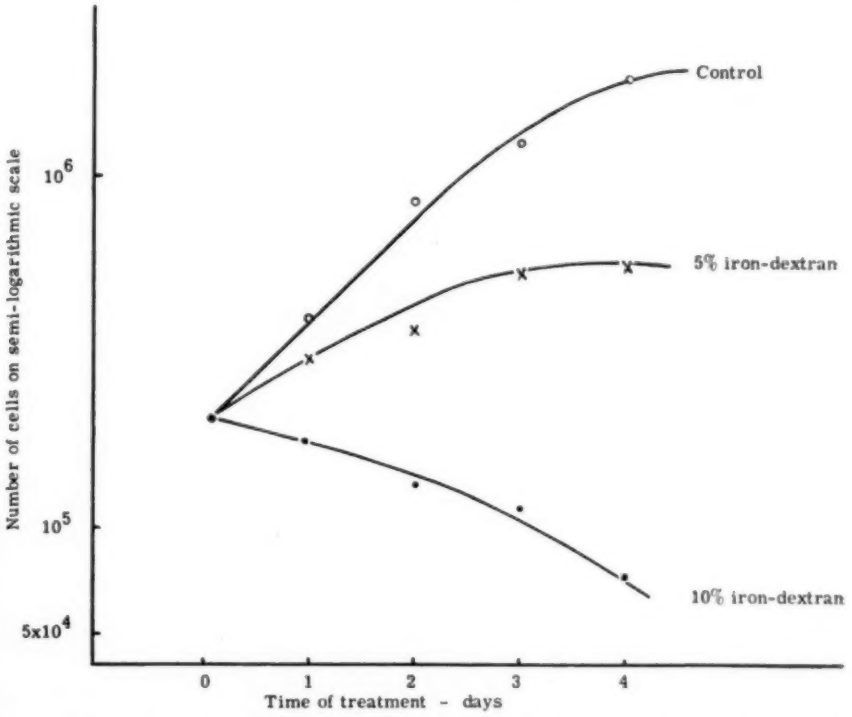


FIG. 2.—Daily estimations of cell population (McCoy fibroblast strain) in relation to content of iron-dextran in the culture medium.

led to cell damage and death, which was gross in degree when the concentration was increased to M/10^4.

Protective effect of increasing serum content of medium

The toxic action of iron-dextran was partially counteracted by increasing the percentage of horse serum in Eagle's medium. There was no difference in the growth of control cultures maintained in 10 or 20 per cent horse serum (Fig. 5) but the toxic action of continued exposure to 2·5 per cent iron-dextran was reduced by 20 per cent serum. Corresponding results were obtained in 10 per cent iron-dextran treated cultures, in that twice as many cells survived after 5 days

treatment in 20 per cent serum compared to 10 per cent serum. When serum was increased to 50 per cent, a toxic action attributable to serum alone became apparent in the control cultures, so that any iron-antagonistic effect at this concentration of serum could not be measured.

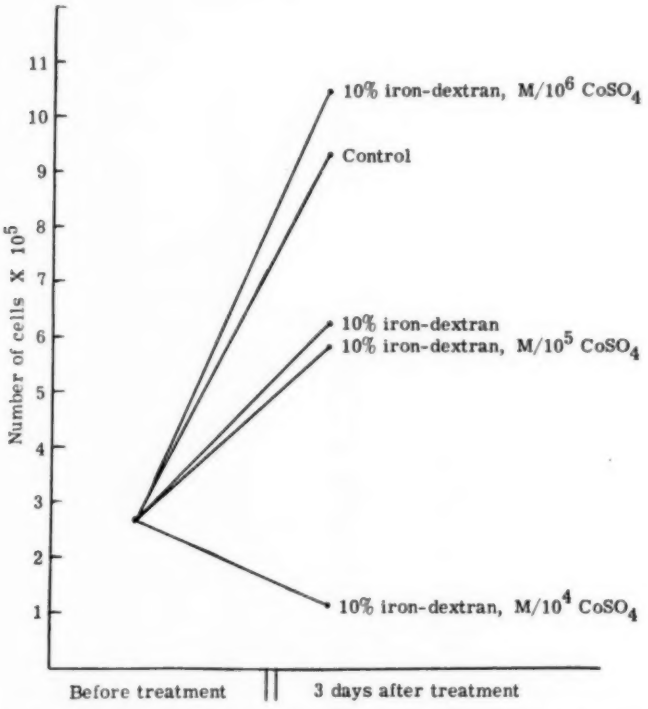


FIG. 3.—Effect of 6 hours' treatment with 10 per cent iron-dextran and cobaltous sulphate on cell growth (McCoy fibroblast strain).

Cytological changes induced by iron-dextran

When cell cultures are treated with iron-dextran and subsequently stained for iron by Perl's method, a noticeable feature is the presence of some cells which show a strongly positive reaction for iron ; an intense blue color is seen throughout the cytoplasm and sometimes the nucelus is also heavily stained.

Treatment followed by fixation and staining at varying times shows that after as little as 2 minutes exposure to 10 per cent iron-dextran, very occasional cells exhibit preferential absorption of iron. Between 10 and 30 minutes there is a slight increase in the number of cells showing this reaction while the depth of iron staining increases to a maximum. There is no difference between cultures treated for 30 minutes and others treated for 120 minutes. Virtually all heavily stained cells show degenerative nuclear changes—pyknosis or karyorrhexis—and

it is concluded that deep diffuse iron staining of these cells is due to breakdown of the normal cell membrane barrier associated with degeneration and death of the cell. At least some of these were degenerating or had died before treatment, as it seems unlikely that iron-dextran could account for nuclear pyknosis after 2 minutes treatment. Killing of cell cultures by heat or cyanide treatment with

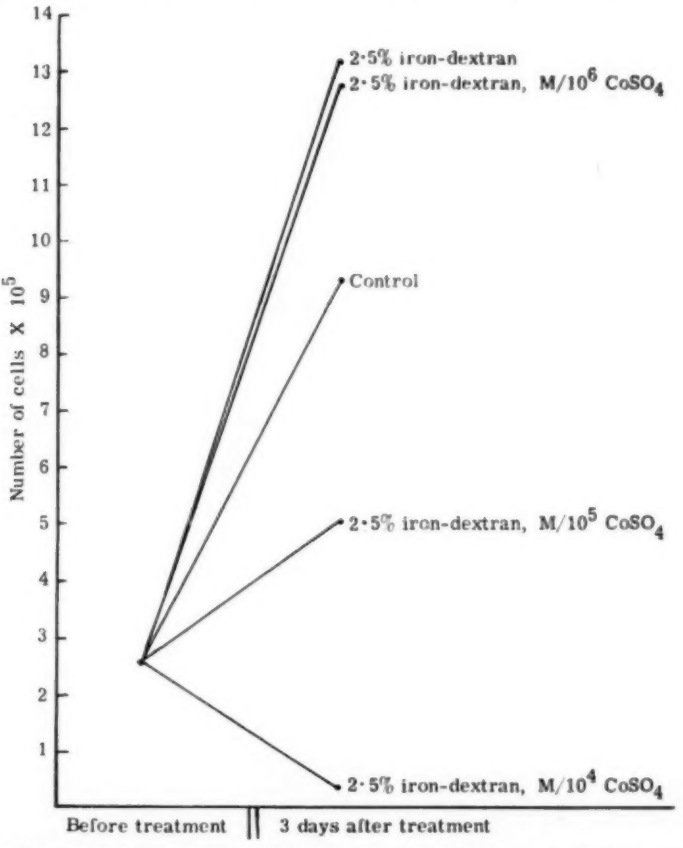


FIG. 4.—Effect of 6 hours' treatment with 2·5 per cent iron-dextran and cobaltous sulphate on cell growth (McCoy fibroblast strain).

subsequent exposure to iron-dextran shows that all the dead cells absorb iron-dextran within 30 minutes.

Six hours of continuous exposure to 10 per cent iron-dextran does not lead to any significant increase in deeply stained degenerate cells, but some cells now contain minute granules of iron in the cytoplasm and the nucleus; this is interpreted as being due to active absorption and storage of iron by living cells. Another feature seen at this time is a change in the appearance of the chromatin network

of many apparently healthy nuclei ; the chromatin tends to become more coarsely stained in May-Grünwald-Giemsa preparations, and assumes a heavily granular character.

When iron-dextran treatment is continued for 24 hours, a number of cells which are morphologically normal are found to be heavily stained with iron. There seems little doubt that this staining reaction is an early sign of death, signifying

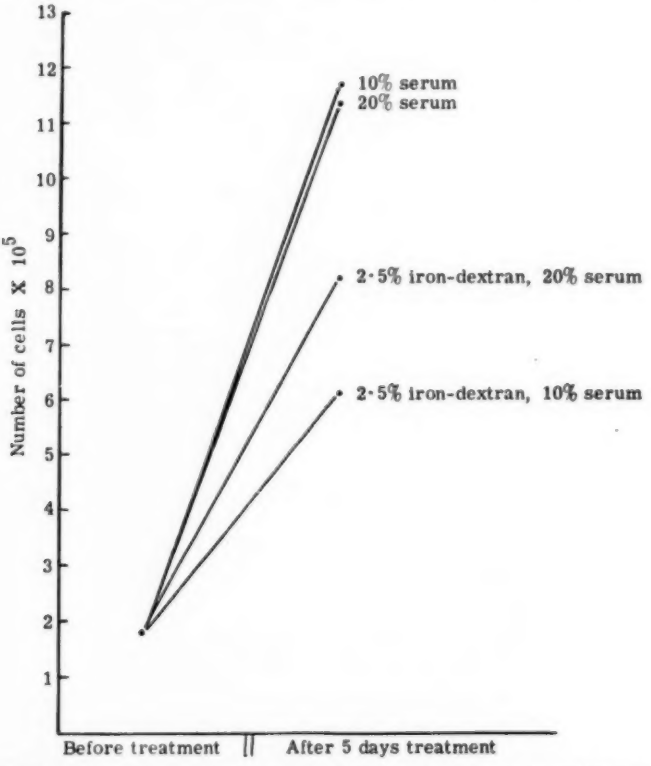


FIG. 5.—Partial protection against toxic action of iron-dextran due to increasing serum content of medium (Fernandez amnion strain).

a loss of integrity in the cell wall which occurs before cytomorphological changes become visible. Correlating with this, there is an increase in the number of cells with obviously degenerate nuclei, so that the proportion of heavily iron-stained cells is around 5 per cent. Mitotic figures are particularly prominent among the cells showing strong iron staining. This impression was confirmed by comparing the mitotic index in control and treated cultures of human lung cells. Controls show a mitotic index of 1·9 per cent while the mitotic index in heavily stained cells alone, in treated cultures, is 11 per cent. This last figure is conservative because many of the heavily stained cells have a dense mass of chromatin which

might be interpreted either as a pyknotic nucleus or as a sticky metaphase plate ; doubtful mitoses were not included in the mitotic count.

With continued exposure to iron for 48 to 72 hours, the total number of cells in the culture lessens and virtually all surviving living cells show granular deposits of iron in the cytoplasm and less marked staining of the nucleus. In some cultures, an additional change becomes evident at 72 hours, namely an increase (up to two-fold) in the number of binucleate and multinucleate cells in treated compared to

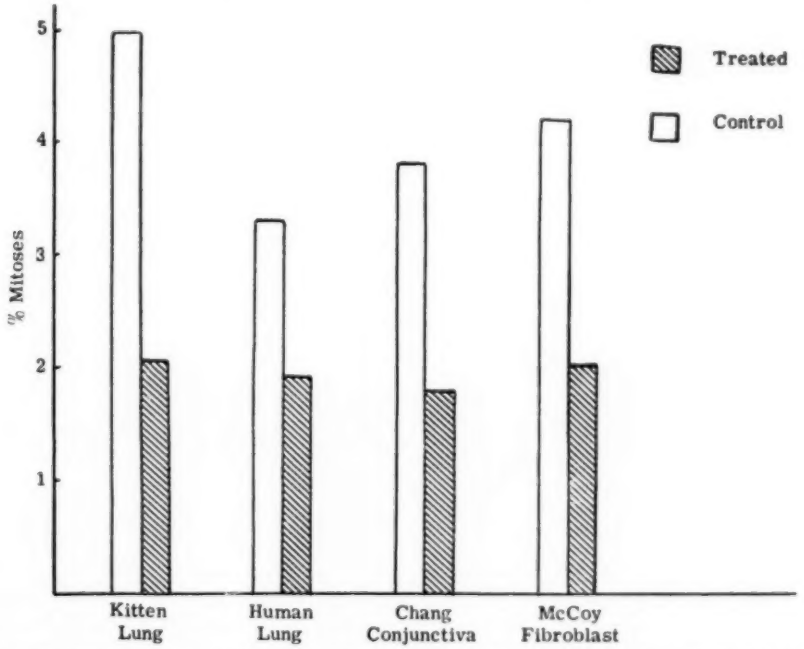


FIG. 6.—Effect of 6 hours' treatment with 10 per cent iron-dextran on the mitotic index in different cell lines.

control cultures. This is presumably a reflection of increased mitotic aberration, which is described below.

At 96 hours, most surviving cells have absorbed a large amount of iron which is distributed in granular fashion throughout the cytoplasm, the appearance being quite different from the diffuse iron staining associated with cell death. The granules of iron show a peculiar localization around the Golgi apparatus (Fig. 8) and it may be that this represents a preferential deposition of iron on the protein and ribonucleic acid which are concentrated at this site.

The effect of iron-dextran on mitosis

Cultures of kitten lung, human lung, Chang conjunctiva and McCoy fibroblasts in Rose chambers were treated with 10 per cent iron-dextran for 6 hours ; after

washing in Gey's balanced salt solution, normal medium was introduced and the cultures were fixed and stained 18 hours later. The mitotic rate of 2000 cells was expressed as a percentage and the results are embodied in Fig. 6. Each total is

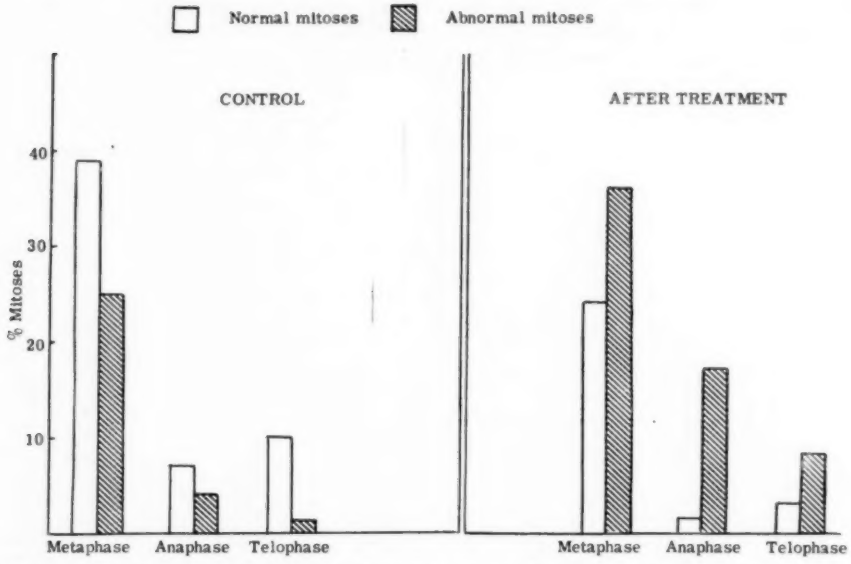


FIG. 7.—Change in ratio of normal to abnormal mitoses after 6 hours' treatment with 10 per cent iron-dextran (human lung strain).

the average of results obtained from 3 separate culture vessels. It will be seen that the treatment reduced the mitotic rate approximately 50 per cent in all cell lines.

Concomitant with this decrease in the number of mitoses, there is an increase in the frequency of mitotic aberrations. Analysis of the same cultures showed a reversal in the proportion of normal to abnormal figures in metaphase, anaphase and telophase (Fig. 7). The most frequent abnormality at metaphase in treated

EXPLANATION OF PLATES

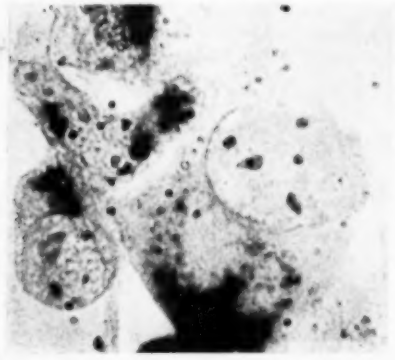
FIG. 8. (a) and (b).—Human lung cells showing dark granular masses of iron in the cytoplasm, after 4 days treatment with iron-dextran. Note how the iron is concentrated around the zone of Golgi, which has become distorted in (a). Perl's method for iron. ×1200.

FIG. 9.—Cell in metaphase showing scattering of chromosomal material. Human lung strain. May-Grünwald-Giemsa. ×1300.

FIG. 10.—Cell in metaphase showing several " sticky " masses of chromosomal material. Human lung strain. M.G.G. ×1300.

FIG. 11.—Cell in anaphase showing bridging due to interlocking of chromosomes. Human lung strain. M.G.G. ×1000.

FIG. 12.—Tetrapolar telophase showing scattering of chromosomes and bridging between two of the daughter cells. Human lung strain. M.G.G. ×1300.



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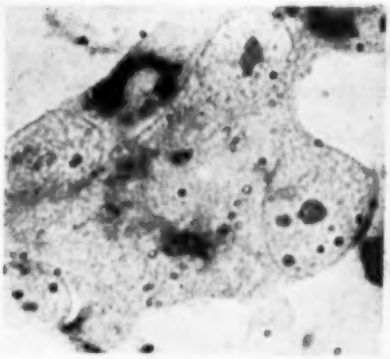

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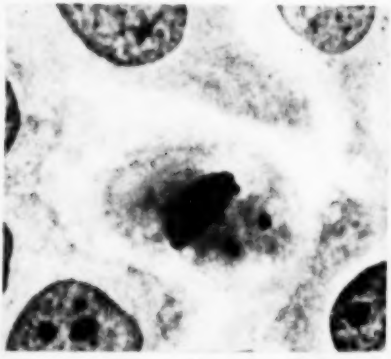

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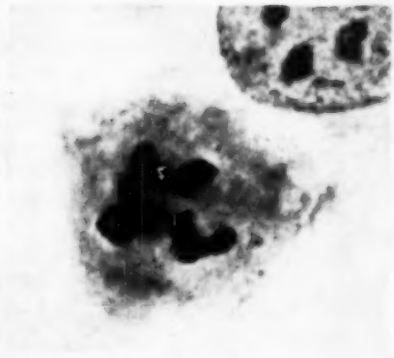

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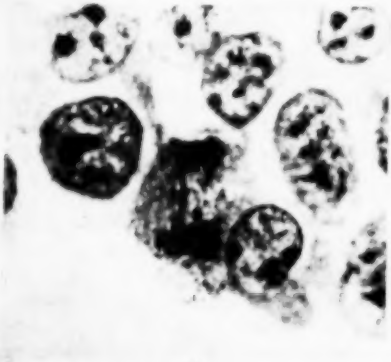

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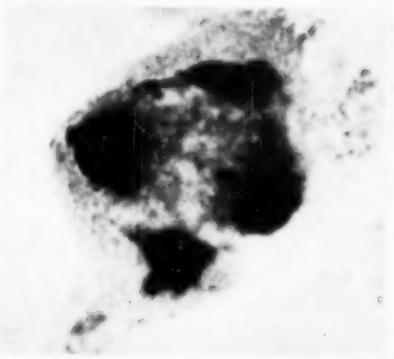

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cultures was scattering of the chromosomal material (Fig. 9), although stickiness and clumping were also increased (Fig. 10). In the anaphase, and telophase treatment with iron-dextran led to an increase in bridging (Fig. 11) and multipolar division (Fig. 12).

DISCUSSION

In considering the toxic effects of introducing excess iron into a cell, the diverse role of metals in the normal life of the cell must be kept in mind. Metals play a necessary role in the working of many biological enzyme systems, notably in the flavoproteins (Nicholas, 1957) and iron in particular is a constituent of cytochrome, peroxidase and catalase. These enzyme systems could be severely disturbed in the presence of excessive amounts of iron.

Metals are not equally distributed in nucleus and cytoplasm, as Mann (1945) has shown in his studies on ram spermatozoa ; iron, copper, and zinc were found in relatively greater quantities in the sperm head, compared to the body and tail. The preponderance of iron in the deoxyribonucleic acid-containing part of the sperm may be related to the special affinity which Albert (1953) has demonstrated between iron and guanosine in his studies on the avidity of naturally occurring substances for trace metals. Further, Kirby (1957) has suggested that deoxyribonucleic acid could form a complex with protein, through a metallic bond between guanine and the carboxyl groups of protein containing aspartic or glutamic acid residues. Steffenson (1955) has also put forward the hypothesis, based on work with *Tradescantia* plants, that bivalent cations are involved in the binding of chromosomal nucleoprotein and therefore play an intimate role in the structural composition of the chromosome.

The present experiments show that continuous exposure of cells to 1 per cent iron-dextran, and short-term exposure to 10 per cent iron-dextran, produces a toxic effect manifested by reduction in growth rate and cytological abnormalities. It is known that several cell constituents are easily oxidized by free oxygen if catalytic traces of iron are present (Albert, 1960) and this, in turn, leads to the formation of hydrogen peroxide which goes on to oxidize more substrate. Such a destructive chain reaction can be broken by traces of cobalt. Therefore the protective influence of cobalt demonstrated in the present study indicates that the toxic action of iron-dextran is due largely to an oxidative mechanism. This conclusion is supported by previous work carried out by Albert and colleagues on the toxic action of another compound of iron, iron-oxine, which exerts a powerful antibacterial action on staphylococcus aureus (Rubbo, Albert and Gibson, 1950). Traces of cobalt in the growth medium counteracted the antibacterial effect of iron-oxine and from this, and other experiments, it was concluded that iron-oxine acts as a catalyst which favours the destructive oxidation of a group (—SH group) on a metabolite or enzyme essential to the cell. Nordbring-Hertz (1955) has studied the effects of oxine on the yeast phase of *Candida albicans* and agrees with Albert that the — SH system of the cell is being affected. The process of cell division in yeast is regulated by the amount of —SH groups (Nickerson and Van Rij, 1949) and it has been suggested that the general tendency of cobalt to counteract the iron-oxine reaction is connected with this cell division mechanism.

Since low molecular weight dextran and oxine have no chemical relationship, apart from ability to complex with iron and other metals, the similar protective

effect of cobalt on iron-dextran and iron-oxine toxicity would suggest that it is iron alone which is the ultimate damaging agent. This conclusion is supported by the ameliorating action of adding additional serum to the medium containing iron-dextran. An increase in serum concentration represents, in effect, an increase in sequestering agents competing for available iron ions, due in part to the metal-binding activity of cysteine and histidine groups in albumin (Tanford, 1952; Gurd and Goodman, 1952). Furthermore, we know that 6 per cent dextran of the type used as a plasma substitute is non-toxic in tissue culture (Pomerat and Overman, 1956). Also, the low molecular weight fraction of dextran is not toxic or carcinogenic *in vivo* (Haddow and Horning, 1960; Richmond, 1960). It may well be, however, that the dextran component plays an important role in the toxic and carcinogenic action of iron-dextran, functioning as a vehicle for easy introduction of iron into the cell. The relative non-carcinogenicity or low carcinogenicity of other compounds of iron (Haddow and Horning, 1960; Richmond, 1959) strengthens this conclusion.

The catalytic formation of intracellular hydrogen peroxide by iron is essentially similar to the cellular change following X-irradiation, which has been reviewed by Dale (1954). X-irradiation causes decomposition of water with release of H and OH radicals, and these can then react together to form hydrogen or hydrogen peroxide with consequent depolymerization of nucleic acid. It is pertinent here to recall the radiomimetic effect of ferrous sulphate and hydrogen peroxide (Boyland and Sargent, 1951) in producing greying of hair, which has been ascribed to the degradation of deoxyribonucleic acid by free hydroxyl radicals. Boyland (1954) suggested that hydrogen peroxide might damage the deoxyribonucleic acid chain by esterifying the phosphate group to give an unstable disubstituted perphosphoric acid derivative. Subsequent hydrolysis would yield a split nucleic acid. Another possible mode of action of peroxide is to react with the amino groups or nitrogen atoms of purines or pyrimidine rings. These disturbances in deoxyribonucleic acid might distort the chromosome sufficiently to produce either functional change manifested by mutation or a visible effect manifested by chromosomal damage.

Mutations have been produced in *E. coli* by iron salts (Demerec, Bertani and Flint, 1951) but experiments carried out in *Drosophila*, quoted by Haddow and Horning (1960) have not shown any mutagenic effect attributable to iron-dextran, suggesting that there is no direct effect on deoxyribonucleic acid. While this may be true for *Drosophila*, the present investigation shows that iron-dextran affects the mitotic apparatus in mammalian cells, at least *in vitro*. These changes have been primary in the sense that they have occurred relatively soon after treatment and have taken the form of an increase in the rate of mitotic abnormalities to which strains of cells in tissue culture are subject. The most frequent abnormalities have been scattering and stickiness in the metaphase, and bridge formations at the anaphase-telophase. Scattering may be attributable to oxidation of —SH groups on the mitotic spindle, interfering with the function of the spindle fibres. The tendency for iron to accumulate in the region of the Golgi apparatus could be important in this respect, for the centrioles, which are responsible for the formation of the spindle apparatus, are located in the Golgi body. Clumping and bridging are characteristic of X-irradiation damage in cells examined soon after treatment (Carlson, 1954). The similar morphological changes seen after iron-dextran treatment may be due to an oxidative mechanism comparable to X-ray damage. It

is pertinent here to recall the investigation by Von Rosen (1954) into the chromosome-breaking action of elements of the periodical system. Using *Pisum* rootlets, he found that complex forming metals, including iron, could give rise to chromosome disturbances of similar morphology to those described in X-irradiation, isotope radiation and treatment with the radiomimetic substance, nitrogen mustard.

Scattering of chromosomes and bridging in anaphase and telophase are known early effects of recognized carcinogens on cells in tissue culture (Biesele, Grey and Mottram, 1956). Boyland (1954) is of the opinion that the mechanisms by which chromosome breakage, mutagenesis and carcinogenesis are brought about are probably similar, being different manifestations of the same effect. Therefore, while the present experiments have been acute in nature, the results shed some light on previous animal experiments demonstrating the carcinogenic potency of iron-dextran.

SUMMARY

In concentrations of 1 per cent and above, iron-dextran complex reduces the growth rate of mammalian cells in tissue culture. A concentration of 10 per cent iron-dextran is sub-lethal.

Traces of cobalt in the growth medium counteract the toxic effect of iron-dextran, suggesting that the mechanism of toxicity is essentially oxidative in nature.

Increasing the concentration of serum in the medium, thereby increasing the concentration of sequestering agents competing for iron ions, leads to a reduction of iron-dextran toxicity. It is concluded that the iron component of iron-dextran complex is the main damaging agent.

With continued exposure to iron-dextran, cells show increasing absorption of iron, which accumulates in the region of the Golgi apparatus. Abnormalities of mitosis are demonstrable, namely, scattering and stickiness in the metaphase and bridging in the anaphase and telophase. Cells undergoing division are particularly liable to degeneration and death.

Many of the cytological changes produced by iron-dextran can be attributed to intracellular oxidation, with iron acting as a catalyst. Comparison is made with the known oxidative mechanism whereby X-irradiation produces its effects on the mitotic apparatus. It is concluded that the acute changes observed in cells treated with iron-dextran *in vitro* form a basis for the development of sarcoma *in vivo*.

This work has been made possible by the help and encouragement provided by Dr. C. M. Pomerat and his staff, to whom I wish to express my gratitude. The investigation has been aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research.

REFERENCES

ALBERT, A.—(1953) *Biochem. J.*, **54**, 646.—(1960) *In* 'Selective Toxicity'. London (Methuen and Co.), p. 162.

BIESELE, J. J., GREY, C. E. AND MOTTRAM, F. C.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 1303.

BONSER, G. M., FAULDS, J. S. AND STEWART, M. J.—(1955) *Amer. J. clin. Path.*, **25**, 126.

BOYLAND, E.—(1954) *Pharmacol. Rev.*, **6**, 345.

UMI

BOYLAND, E. AND SARGENT, S.—(1951) *Brit. J. Cancer*, **5**, 433.
CARLSON, J. G.—(1954) *In* 'Radiation Biology,' *I*, Part 2. New York (McGraw-Hill Book Co.), p. 799.
DALE, W. M.—(1954) *Ibid.*, *I*, Part 1, p. 255.
DEMEREC, M., BERTANI, G. AND FLINT, J.—(1951) *Amer. Nat.*, **85**, 119.
FAULDS, J. S.—(1957) *J. clin. Path.*, **10**, 187.
Idem AND STEWART, M. J.—(1956) *J. Path. Bact.*, **72**, 353.
GURD, F. AND GOODMAN, D.—(1952) *J. Amer. chem. Soc.*, **74**, 670.
HADDOW, A. AND HORNING, E. S.—(1960) *J. nat. Cancer Inst.*, **24**, 109.
KENNAWAY, E. L. AND KENNAWAY, N. M.—(1936) *J. Hyg., Camb.*, **36**, 236.
KIRBY, K. S.—(1957) *Biochem. J.*, **66**, 495.
MCDOUGALL, A.—(1956) *J. Bone Jt. Surg.*, **38**B, 709.
MANN, T.—(1945) *Biochem. J.*, **39**, 451.
NICHOLAS, D. J. D.—(1957) *Nature, Lond.*, **179**, 800.
NICKERSON, W. J. AND VAN RIJ, N. J. W.—(1949) *Biochim. biophys. Acta*, **3**, 461.
NORDBRING-HERTZ, B.—(1955) *Physiol. Plant.*, **8**, 691.
POMERAT, C. M. AND OVERMAN, R. R.—(1956) *Z. Zellforsch.*, **45**, 2.
RICHMOND, H. G.—(1957) *Scot. med. J.*, **2**, 169.—(1959) *Brit. med. J.*, i, 947.—(1960) *In* 'Cancer Progress'. London (Butterworth and Co.), p. 24.
VON ROSEN, G.—(1954) *Hereditas*, **40**, 258.
RUBBO, S. D., ALBERT, A. AND GIBSON, M. I.—(1950) *Brit. J. exp. Path.*, **31**, 425.
SIDDONS, A. H. M. AND MACARTHUR, A. M.—(1952) *Brit. J. Surg.*, **39**, 542.
STEFFENSEN, D.—(1955) *Genetics*, **40**, 598.
TANFORD, C.—(1952) *J. Amer. chem. Soc.*, **74**, 211.
WARREN, S. AND DRAKE, W. L.—(1951) *Amer. J. Path.*, **27**, 573.

THE CARCINOGENIC ACTION OF CARRAGEENIN IN RATS

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ROBERTSON and Schwartz (1953) showed that " carrageenin ", (a sulphated polygalactose made from the sea weed, Irish moss, *Chondrus crispus*,) when injected subcutaneously into guinea-pigs would induce the proliferation of fibroblasts and the formation of a connective tissue granuloma. This technique was used to study the biochemistry of collagen formation by Jackson (1956*a* 1956*b*, 1957), Slack (1956, 1957, 1958), Robertson and Hinds (1956), Robertson, Hiwett and Herman (1959). The histology of the lesions produced has been described by Williams (1957) and Benitz and Hall (1959).

Walpole, Roberts, Rose, Hendry and Homer (1954) showed that several simple monofunctional, N-acylethyleneimines would produce tumours in rats and mice when given by repeated subcutaneous injections. In no case, however, was a tumour produced by a single dose of the carcinogen. It therefore appeared of interest to see if a single dose of an ethyleneimine would induce a tumour if it was injected into the mass of young fibroblasts produced by carrageenin. Young female rats were therefore given an injection of carrageenin into the left flank and injections of acylethyleneimine into both right and left flanks. The injection of carcinogen into the left flank was placed in the centre of the carrageenin induced granuloma and was timed to coincide with its height. A large number of rats produced sarcomas—all on the left side—but control rats, injected with carrageenin only, also produced sarcomas. Although no synergy between carcinogen and carrageenin could be demonstrated, the changes produced by the carrageenin in the mammary glands of the rats, and the changes leading to the formation of the tumours are of interest and are described in this report.

METHODS

Female Wistar rats 60 days old and between 100 to 130 g. in weight were injected into the left flank with 5 ml. of 1 per cent w/v carrageenin in 0·9 per cent salt solution. Three days later they were injected into the right flank with 1·5 mg. of caproylethyleneimine in 0·15 ml. arachis oil, and on the 7th day a similar quantity of caproylethyleneimine was injected into the middle of the carrageenin induced mass in the left flank. Some preliminary experiments had confirmed that 7 days after the injection of carragenin there were numerous young fibroblasts present in the mass of new fibrous tissue which had been induced. The two doses of ethyleneimine were given on separate days to avoid loss of rats from the toxicity of this reactive compound. In all four batches of rats were used :

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Preliminary Experiment :

Group A 47 injected carrageenin left side and caproylethyleneimine on both sides.

Main Experiment :

Group B 28 injected carrageenin left side and caproylethyleneimine on both sides.

Group C 28 injected carrageenin left side and acetylethyleneimine on both sides.

Group D 43 injected carrageenin left side and saline on both sides.

When the rats developed tumours or were ill from other causes they were killed and the mammary tissue from both sides was taken for section. Any tumour or other lesion was also examined by routine histological techniques.

Carrageenin (lot 8–9–55) was kindly provided by Mr. L. Stoloff of the Sea Plant Chemical Corporation. 5 g. were dissolved in 500 ml. of 0·9 per cent NaCl and the solution was sterilised by heating in a water bath for 30 minutes.

The caproylethyleneimine and the acetylethyleneimine were kindly provided by Dr. F. L. Rose of the Imperial Chemical Industries. It was already made up in solution 10 mg./ml. w/v in arachis oil.

RESULTS

In the preliminary experiment (group A) 16 rats were killed 307 days after the injection of carrageenin. At autopsy it was noted that the mammary tissue on the left side differed markedly in appearance from that on the right side. The right side showed a little, rather thin mammary tissue embedded in normal subcutaneous fat, typical of a rather senile, resting mammary gland. On the left side the mammary tissue was much thicker, there was dilatation of the small vessels, the fat was yellow or brown and was sometimes creamy in consistency and the tissue was adherent to the skin. Because of these changes the remaining animals were allowed to survive until they had produced tumours or were ill or died. No rats produced sarcomas on the right side, but 9 out of 18 rats surviving more than 400 days produced sarcomas on the left side. The main experiment was therefore set up consisting of groups B, C and D (Table I). In this experiment all the sarcomas arose in the left flank, but it showed the unexpected result that carrageenin alone would induce sarcomata. We were not able to prove by the detailed statistical analysis, given below, that a single dose of an acylethyleneimine given into the carrageenin-induced granuloma had a significant synergistic effect in producing more sarcomas or shortening the induction period.

Statistical analysis

Table I shows the tumour incidence and the mean induction time of tumours of the 4 groups of animals surviving more than 400 days. Since experiment A was terminated after 650 days, the mean induction times of tumours for groups B, C and D up to 650 days were also computed to allow comparison with experiment A. The table shows that there are no statistically significant differences between the three groups B, C and D after 650 days. The principal difference

TABLE I.—*Tumour Incidence and Mean Induction Times*

Treatment—	Preliminary experiment Group A	Main experiment Group B	Main experiment Group C	Main experiment Group D	Groups A and B pooled
To right flank	Caproylethyleneimine	Caproylethyleneimine	Acetylethyleneimine	Saline	Caproylethyleneimine
To left flank	Carrageenin + caproylethyleneimine	Carrageenin + caproylethyleneimine	Carrageenin + acetylethyleneimine	Carrageenin + saline	Carrageenin + caproylethyleneimine
Number of rats with sarcomas on right side	0	0	0	0	0
Number of rats surviving more than 400 days	18	23	26	39	41
Number of rats developing sarcomas on left side in 650 days	9	3	3	6	12
Mean induction time truncated at 650 days and standard error	590·6 ± 20·8	635·5 ± 10·5	637·4 ± 6·4	640·6 ± 4·7	615·2 ± 11·5
Number developing sarcomas on left left side in 825 days	—	5	9	11	14
Mean induction time truncated at 825 days	—	784·3 ± 22·1	749·4 ± 20·9	766·0 ± 17·1	724·3 ± 23·1

TABLE II.—*Expected Numbers Developing Tumours*

	Group A	Group B	Group C	Group D	Group A and B pooled
Number of rats surviving more than 400 days	18	23	26	39	41
Number of sarcomas developing in 650 days	9	3	3	6	12
Expected number per 1000 developing sarcomas in 650 days and standard error	653·8 ± 140·8	150·4 ± 81·0	135·6 ± 73·9	199·3 ± 73·4	350·2 ± 83·9
Expected number in experiment developing sarcomas in 650 days	11·8	3·5	3·5	7·8	14·4
Number developing sarcomas in 825 days	—	5	9	11	14
Expected number per 1000 developing sarcomas in 825 days	—	635·9 ± 227·4	536·7 ± 140·0	547·2 ± 136·4	721·5 ± 175·6
Expected number in experiment developing sarcomas in 825 days	—	14·6	14·0	21·3	29·6

Note : The expected numbers above refer to the expected numbers per thousand that would develop sarcomas if there were no deaths from other causes.

in mean induction times at 650 days is between group A and the other three groups. The difference between groups A and B is not significant at the 5 per cent level. The difference between groups A and C, and A and D exceed twice their standard errors, but it is not possible to assess the precise significance of their difference owing to uncertainty as to the degrees of freedom that should be attached to the standard errors. Sukhatme's d test (Fisher and Yates, 1953, Table V. 1) indicates, however, that the differences are near the 5 per cent level. Further the larger standard deviation of group A (which is approximately given by $\sqrt{9} \times 20{\cdot}8 = 62{\cdot}4$) compared with the other groups suggests that the form of the distribution of induction times for group A may be different from that of groups B, C and D.

After 825 days, there is no statistically significant difference between the mean induction times for groups B, C and D. If the results from groups A and B are pooled there is no statisticant difference between the mean induction time for this group and the remaining two groups C and D.

Table II shows, assuming no deaths from other causes, the number of rats per thousand that would be expected to have developed tumours after both 650 and 825 days. After 650 days there is no statistically significant difference between groups B, C and D, but there is between these groups and group A, the latter developing more tumours per thousand. After 825 days there is again no statistically significant difference between groups B, C and D.

The figures were computed according to Irwin (1949)

Macroscopic changes induced by carrageenin

A mass was palpable in the region of the injection site for about two weeks. Loss of hair was noticed over the injection site commencing about three weeks after the injection and persisting in many cases for many months. When tumours began the palpable mass usually increased very rapidly in size. In most cases it was necessary to kill the animal within three weeks of first detecting the tumour, but sometimes a small mass was felt for some weeks and then suddenly increased rapidly in size. The changes in the mammary tissue at autopsy included :— a visible increase in the amount of mammary tissue on the left side, a fluid or creamy consistency of the fat, numerous small vessels in the injection area and adherence of the mass to the skin.

Histological appearances of the changes induced by carrageenin

The essential changes induced by carrigeenin can be described under 6 headings.

1. *The early changes.*—These consist of an accumulation of polymorphonuclear leukocytes, macrophages with phagocytosed polysaccharide and fibroblasts. Fig. 1 shows the appearance one week after the injection and shows many macrophages and numerous young fibroblasts. Later mature collagen fibres are in greater evidence. The early changes have been described in detail by Williams (1957) and Benitz and Hall (1959) and need not be further elaborated here.

2. *Degeneration of mammary gland epithelium* was seen in progress even more than two years after the carrageenin injection. Fig. 2 shows the typical appearance of the unaffected mammary tissue on the right side. Note a small cluster of gland alveoli separated by much adipose tissue from its nearest neighbour. Note also the thin walled normal capillaries. Fig. 3 shows that the changes

induced by carrageenin are still progressing 813 days after injection. Note in the centre of the field mammary epithelial cells in process of degeneration. In other parts of the field the empty spaces represent gland alveoli which have degenerated. There is a great increase of the intra-lobular fibrous tissue.

3. *Invasion of degenerated gland alveoli by fibroblasts.*—Fig. 4 shows a good example of this in the lower part of the field ; note how the space left by a mammary gland alveolus which has degenerated has now been filled with a group of fibroblasts. A somewhat earlier stage of this process is seen near the middle of the right margin of this picture.

4. *Thickening of the walls of the capillaries and arterioles.*—This change which can be seen in Fig. 3 and 4 is illustrated more clearly in Fig. 5, 6 and 7. Fig. 5 is stained with methyl violet and shows the thick layer of hyaline material surrounding the endothelial lining of the capillary. There are many mast cells present immediately outside this hyaline material. With methyl violet the hyaline material is faintly metachromatic and the mast cells stain deep blue. Fig. 6 is from the same block and is stained with P.A.S. At the bottom of the picture the thickened capillaries and the mast cells are very clear,. In the top right hand corner, there are capillaries with walls as much as 4 times as thick as the diameter of the capillary lumen. Fig. 7, stained with haematoxylin and eosin, shows a small arteriole with a greatly thickened wall running in a tortuous spiral diagonally across the field. Other thickened vessels can be easily identified in other parts of this picture.

5. *Involvement of small nerves in the fibrous tissue.*—The fibrous tissue replacement of the mammary tissue appears to enmesh many small nerves. Fig. 8 shows a typical field. Fig. 9 shows four of the changes induced by carrageenin : —the cells lining the mammary gland alveoli have become pale staining, with vacuolated cytoplasm and small pyknotic nuclei ; there is definite replacement of gland cells by fibroblasts in the central alveolus ; the capillary walls are thickened and a small nerve has been caught in the area of fibrosis.

6. *Sarcomatous changes.*—Zones of precancerous proliferation and early sarcomatous changes were seen in some of the sections. Fig. 10 is from a zone 1 to 2 mm. across which contains many large cells with heavy staining and abnormal nuclear formations. Fig. 11 shows part of an early fibroblastic sarcoma. Most of the large tumours were of this type but some were fibro-sarcomata containing collagen fibres stainable by Van Gieson and some were more anaplastic in type. One typical myxosarcoma was seen.

One rat with a large fibroblastic type sarcoma had bilateral deposits at the lower poles of both kidneys, see Fig. 12 which shows the tumour cells infiltrating between the kidney tubules. A lymphosarcoma of the thymus was also noted in another rat.

DISCUSSION

The original purpose of the experiment was to show whether a single dose of an acylethyleneimine, a simple but short acting carcinogen, would produce a tumour if placed in contact with young, actively growing fibroblasts. The whole basis of the experiment was invalidated by the quite unexpected finding that carrageenin by itself would produce sarcomata. Careful statistical analysis of the results did not show a significant synergistic activity of the single dose of ethyleneimine and the carrageenin.

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The long-lasting changes produced by the carrageenin were also quite unexpected. Williams (1957) and Benitz and Hall (1959) described the histological changes produced by carrageenin in the first 28 days following injection. The author has been unable to find any description in the literature of the late effects of injected carrageenin. The degenerative changes in mammary gland epithelium and replacement by fibroblasts are what might have been anticipated but the curious changes in the small vessels were a surprise. The small vessels appear like pipes with thick walls, the hyaline material stains slightly pink with methyl violet, red with periodic-Schiff, eosinophil with haematoxylin and eosin and blue with the picro Mallory stains. Frequently there are many mast cells surrounding these vessels. It is possible that the chronic changes in the mammary gland are due to the interference with its blood supply, but macroscopically the carrageenin-injected tissue appears to be much more vascular than the control gland.

The loss of hair over the injection site is also something of a mystery. It might be due to irritation and scratching by the animal—irritation due to alterations of blood supply or the inclusion of nerve fibres in the mass of fibrous tissue. On the the other hand the hair loss might be due to the interference with the subepidermal layer of elastic tissue and hair follicles. Williams (1957) notes that intradermal injection of carrageenin in the guinea-pig produced after 5 days a degeneration of the collagenous fibres in the dermis, and despite partial remodelling in the later stages the dermis suffered a substantial loss of its collagenous tissue. My clinical impression is that some of the changes in the skin

EXPLANATION OF PLATES

FIG. 1.—Subcutaneous tissue of a rat 7 days after injection of carrageenin only; note the numerous macrophages and fibroblasts. H. & E. ×570.

FIG. 2.—Control mammary tissue (on right side) 722 days after start of experiment. Note thin walled capillary and much interlobular adipose tissue. Picro Mallory. ×475.

FIG. 3.—Degeneration of mammary gland epithelium with increase of intralobular fibrosis and hyaline thickening of capillary walls seen 454 days after injection of carrageenin + caproylethyleneimine. Picro Mallory. ×360.

FIG. 4.—Degenerated mammary gland epithelium being replaced by fibroblasts, see gland alveolus at bottom centre, also hyaline thickening of capillary walls, 545 days after injection of carrageenin + acetylethyleneimine. Picro Mallory. ×360.

FIG. 5.—Hyaline thickening of capillary walls which are surrounded by mast cells, same rat as Fig. 3, 454 days after injection of carrageenin + caproylethyleneimine. Methyl violet. ×360.

FIG. 6.—From the same rat as Fig. 3 and 5, stained with PA.S. ×360.

FIG. 7.—Tortuous arterioles and capillaries with hyaline thickening of their walls, 803 days after injection of carrageenin, only. H. & E. ×360.

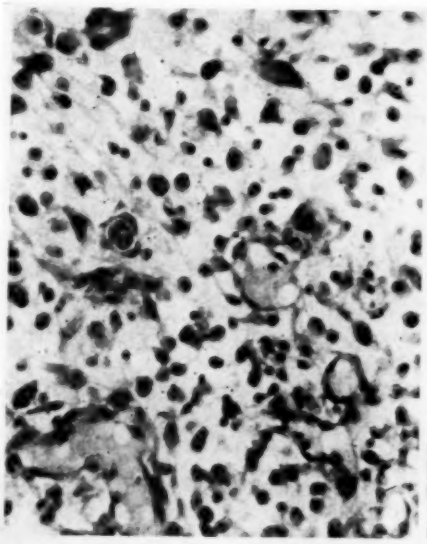
FIG. 8.—A number of fine nerves caught in the fibrous tissue replacing the mammary tissue 813 days after injection of carrageenin only. Picro Mallory. ×360.

FIG. 9.—Typical carrageenin changes—pale vaculated mammary gland epithelium with pyknotic nuclei with replacement by fibroblasts, upper centre; there is a small nerve caught in the process and some hyaline thickening of capillary walls 562 days after injection of carrageenin + acetylethyleneimine. Picro Mallory. ×360.

FIG. 10.—Section from a small zone 1–2 mm. in diameter in which sarcomatous transformation has taken place, 545 days after injection of carrageenin + acetylethyleneimine. H. & E. ×360.

FIG. 11.—Typical fibroblastic type of sarcoma 618 days after carrageenin injection + caproylethyleneimine. The tumour 40 × 15 × 15 mm. in size appeared 28 days previously. H. & E. ×570.

FIG. 12.—Tumour invading right kidney. There is a pleomorphic-cell sarcoma in left flank 722 days after injection of carrageenin + acetylethyleneimine. H. & E. ×360.



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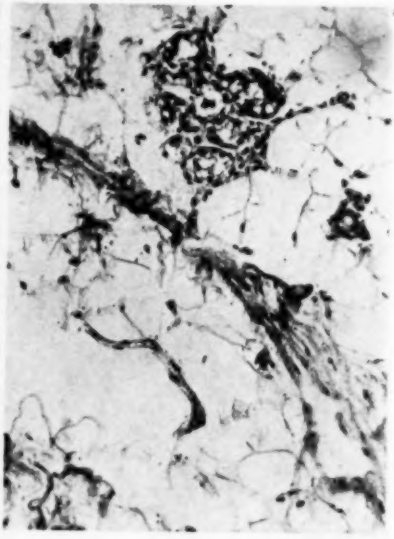

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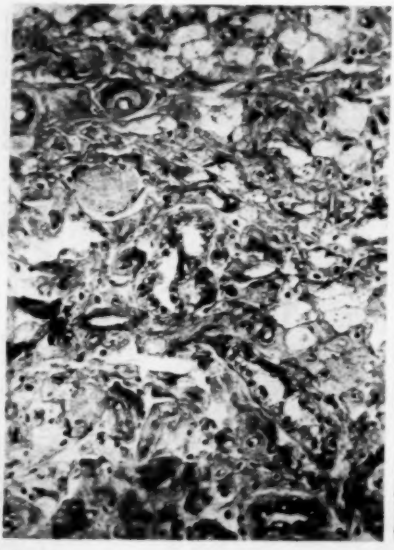

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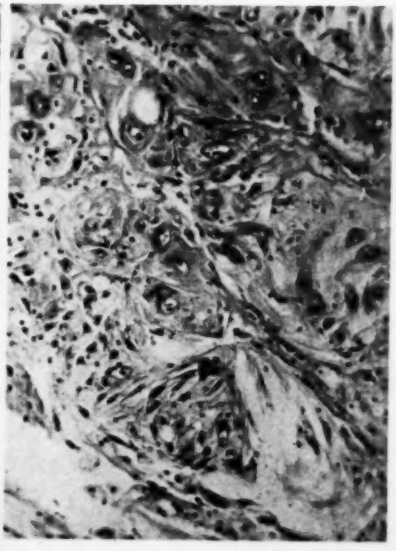

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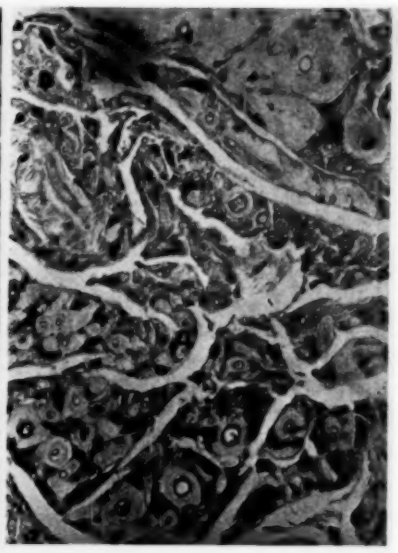

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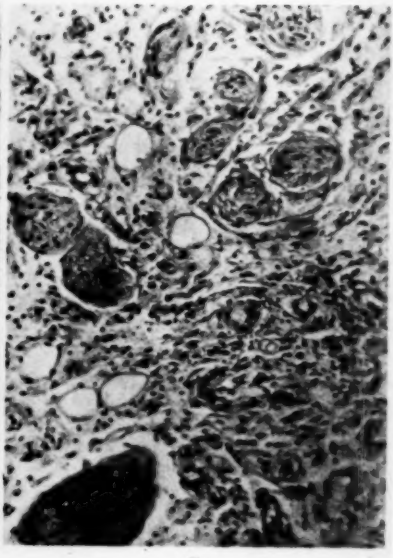

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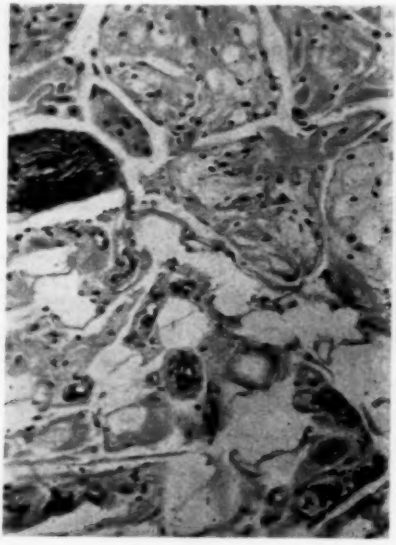

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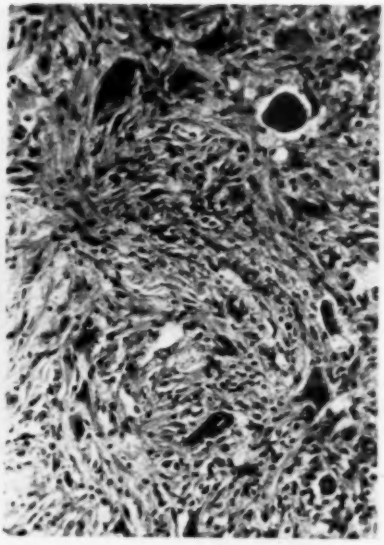

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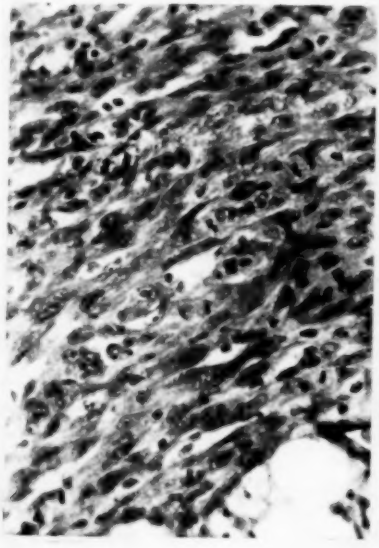

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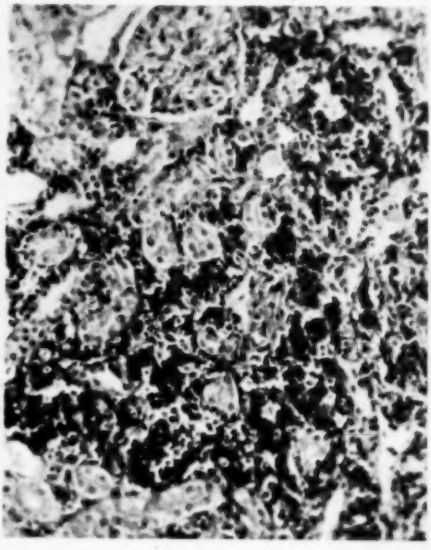

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in the rats were the result of scratching. Not infrequently scratch marks could be seen on the area showing loss of hair, and in a few rats shallow ulcers or scabs were present from time to time. At autopsy the skin appeared to be thin and definitely adherent to the mammary tissue and the vessels immediately beneath the epidermis were engorged. The loss of hair persisted for many months.

The precise aetiology of the tumours is equally mysterious. There are several possibilities and these are not mutually exclusive :

1. Often repeated trauma due to irritation and scratching.
2. Anoxia due to the changes in the vessels.
3. Induction of a tumour in relation to material which cannot be removed from the tissues e.g. tumours induced by plastic films (Oppenheimer, Stout, Oppenheimer and Willhite, 1957).
4. A chronic or repeated stimulus to the production of fibroblasts due to the continued presence of carrageenin acting as a fibroblastic ground substance, or due to the damage which it has caused.
5. The fibrous tissue response to an abnormal ground substance may not be a normal response. For instance Vasiliev (1959) noted that the connective tissue reaction to a pellet containing dimethylbenzanthracene or methylcholanthrene was abnormal—" a distorted fibrogenesis " in which after 3 or 4 months multiple sarcomatous focci appear.

SUMMARY

1. Young female rats were injected into the right flank with a single dose of an acylethyleneimine and into the left flank with a sulphated polygalactose, carrageenin, followed 7 days later by an injection of the acylethyleneimine into the carrageenin induced mass of fibroblasts.

2. Control rats were injected with carrageenin into the left flank and with saline into both flanks.

3. No rats produced sarcomas on the right side, but 11 out of 39 control rats developed a sarcoma on the side injected with carrageenin.

4. 5 out of 23 rats injected with carageenin + caproylethyleneimine and 9 out of 25 rats injected with carageenin + acetylethyleneimine developed sarcomas on the left side, but there was no statistical evidence that the acylethyleneimine had increased the incidence of tumours or shortened the induction period, but in a preliminary experiment using caproylethyleneimine more sarcomata were produced at an earlier date.

5. Carrageenin induced a degeneration of mammary gland epithelium with fibrous tissue replacement and a hyaline thickening of capillary walls. Numerous small nerves were caught in the fibrosis and there was prolonged loss of hair over the injection site.

I wish to thank Dr. F. L. Rose, F.R.S., of Imperial Chemical Industries Ltd., who kindly supplied the capryol and acetylethylenenimines, and Mr. L. Stoloff of the Seaplant Chemical Corporation for the carrageenin. I am greatly indebted to Mr. R. G. Carpenter and Miss A. Young of the Department of Human Ecology, University of Cambridge, for the statistical analysis of data and to Miss N. C. Burr of the Department of Pathology for the care of the animals and for technical assistance.

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REFERENCES

BENITZ, K.-F. AND HALL, L. M.—(1959) *Proc. Soc. exp. Biol., N.Y.*, **102**, 442.
FISHER, R. A. AND YATES, F.—(1953) Statistical Tables for Biological, Agricultural and Medical Research.' 3rd Edition. London. (Oliver & Boyd), p. 44, Table v, i.
IRWIN, J. O.—(1949) *J. Hyg.*, **47**, 188.
JACKSON, D. S.—(1956*a*) *Biochem. J.*, **62**, 25P.—(1956*b*) *Ibid.*, **64**, 8P.—(1957) *Ibid.*, **65**, 277.
OPPENHEIMER, B. S., STOUT, A. D., OPPENHEIMER, E. T. AND WILLHITE, M.—(1957) *Proc. Amer. Ass. Cancer Res.*, **2**, 237.
ROBERTSON, W. VAN B. AND HINDS, H.—(1956) *J. biol. Chem.*, **221**, 791.
Idem, HIWETT, J. AND HERMAN, C.—(1959) *Ibid.*, **234**, 105.
Idem AND SCHWARTZ, B.—(1953) *Ibid.*, **201**, 689.
SLACK, H. G. B.—(1956) *Biochem. J.*, **64**, 7P.—(1957) *Ibid.*, **65**, 459.—(1958) *Ibid.*, **69**, 125.
VASILIEV, J. M.—(1959) *J. nat. Cancer Inst.*, **23**, 441.
WALPOLE, A. L., ROBERTS, D. C., ROSE, F. L., HENDRY, J. A. AND HOMER, R. F.—(1954) *Brit. J. Pharmacol.*, **9**, 306.
WILLIAMS, G.—(1957) *J. Path. Bact.*, **73**, 557.

A HISTOLOGICAL STUDY OF THE LATENT PERIOD OF HEPATOMA GRAFTS

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In their study of transplanted hepatomas in inbred mice Andervont and Dunn (1952) noted a long latent period before grafts became palpable and that the majority of their grafted tumours grew in only a proportion of recipients in the same strain of inbred mice. They considered that non-genetic factors were operating against successful establishment of transplantable primary hepatomas. They also pointed out that the long latent period might account for failure of some of the grafts to take before the experiment was ended and that studies during this period of slowly developing tumours might be helpful in solving the problem, in clinical medicine, of how tumour cells survive in cases where recurrences or metastases occur many years after removal of a primary tumour.

Our experiments have been carried out to study the histological appearances of slowly growing hepatoma grafts each week during the latent period, which was taken as the time between grafting and the detection of a palpable tumour by weekly examination. The tumour studied is a spontaneous hepatoma from a 23 months old RIIIf male breeder and was originally grafted by Dr. B. D. Pullinger, in this Department. A large, irregular slightly nodular swelling was found in the left posterior lobe of the liver. It was paler in patches than the rest of the liver which appeared normal. There were some very large vessels on the surface of the tumour making grooves in it. On histological examination the tumour is seen to be a trabecular hepatoma. It is composed of cords of liver-like cells in which the cytoplasm is abundant and the nucleus has the size, appearance and prominent nucleoli of normal liver cells. Mitoses are fairly frequent (66 per 100 high power fields). Some unusual intracytoplasmic inclusion bodies are present (Laird, 1955 ; Laird and Head, 1956).

The hepatoma was grafted subcutaneously into 10 male RIIIf mice for the first passage. Half of the grafts grew after a latent period of 5 to 14 months before they became palpable. In the remaining 5 mice, discrete groups of apparently viable hepatoma cells were found in sections of the site of injection after 8 to 12 months. The tumour was therefore considered a suitable one in which to study the histological appearances during the latent period. In the second generation, female as well as male mice were grafted. One of 2 males and none of 5 females killed after one year had successful grafts. In the third generation 6 of 7 male mice had palpable grafts. In the fourth generation 6 of 8 males and none of 13 females had successful grafts (Head and Laird, 1957). The higher incidence of primary spontaneous hepatomas in male than in female mice has previously been

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noted by various authors (Burns and Shenken, 1940 ; Andervont, 1950 ; Agnew and Gardner, 1952).

The 5th, 6th and 8th transplant generations have been studied in the present experiment (Head and Laird, 1958 and 1959). The 7th generation transplants were used in another experiment.

The appearances of the grafted tumour are very similar to those of the original spontaneous primary hepatoma. Parts of it are very liver-like, both on naked eye and histological examination. The larger grafts are usually pale brown and often have cystic, haemorrhagic or necrotic areas. Microscopically they are composed of cords of cells in which the cytoplasm is abundant and the nucleus has the size and appearance, with prominent nucleoli and well defined nuclear membrane, of normal liver parenchymal cells. Although mitoses are not very frequent and cells are well differentiated they are considered to be malignant tumours, as metastases have been found in the lungs of mice in previous experiments with this same type of slowly growing hepatoma. The vascular supply to the grafts is usually very good and enlarged vessels are seen around them.

MATERIAL AND METHODS

Four groups of mice were grafted :

1. 51 male mice RIIIf and ($C_3Hf \times RIIIf$) $\times$ RIIIf F_1 with a 5th generation transplant of the grafted tumour.
2. 21 female and 5 male mice ($C_3Hf \times RIIIf$) $\times$ RIIIf F_1 with a 6th generation transplant.
3. 14 female and 5 male mice RIIIf and ($C_3Hf \times RIIIf$) $\times$ RIIIf F_1 with a 6th generation transplant.
4. 24 male mice RIIIf and ($C_3Hf \times RIIIf$) $\times$ RIIIf F_1 with an 8th generation transplant.

First Series : 51 *male mice*

25 RIIIf and 26 ($C_3Hf \times RIIIf$) $\times$ RIIIf F_1 mice aged 3 months were used They were anaesthetised with Bromethol, a small mid-line incision was made and a portion of graft inserted into a pocket dissected in the subcutaneous tissue of the right flank with a pair of blunt pointed forceps. The tumours to be grafted were removed aseptically from the donor and placed in a sterile Petri dish. Portions of the tumour were chosen which were non-necrotic, non-fatty and non-haemorrhagic. The tissue was kept from becoming dry by keeping the lid of the dish moist with sterile saline. Approximately equal portions of tissue were grafted. The mice were numbered by ear-marking and one pure line and one back cross were chosen at random to be killed each week, after grafting. Three mice were killed in the 1st week, and three in the 15th week (in each case the third mouse was ill), two additional mice were killed at the 11th week, as the first two had palpable tumours. A third mouse died at the 16th week and only one was killed at the 21st week in order to leave five mice alive till they died or had large tumours. This was done in order to compare the number of palpable tumours at the later stage with the various appearances of the graft sites in the earlier stage of the experiment, to obtain further information about the length of the latent period and to find whether or not metastases occurred from the large slowly growing grafts.

The site of grafting could be made out at autopsy, when the skin was reflected, either macroscopically or by using a dissecting microscope with $\times 7 \times 10$ magnification. It appeared either as tiny white nodules or as a small pigmented area. The graft and surrounding tissue were pinned out on cork, fixed in Bouin's fluid (sometimes stained in bulk) and then prepared for histological examination. Spiral sections were stained with haematoxylin and eosin or haemotoxylin eosin heloxine tartrazine. The white nodules consisted of small encapsulated groups of hepatoma cells. Sections were stained to try to identify the pigment in some of the hepatoma cells and in the surrounding tissues.

Groups of hepatoma cells were found in one or more of the mice examined each week until the 18th, with the exception of the 7th and 9th weeks when no graft could be found. In mice killed from the 19th to the 21st week no growing hepatoma cells were seen. There was no apparent difference in the grafts in the RIIIf and the back cross mice so they will be described together.

First week.—In one mouse which was killed because it was ill, vacuolated hepatoma cells lying singly and in small groups were found in a small fibrous nodule about 1 mm. diameter (Fig. 1). In two other mice killed after 1 week, although the site of the graft could be seen macroscopically as a small yellow nodule, no liver cells were found microscopically.

Second week.—In one mouse the graft site was seen through the dissecting microscope as an area of tiny white nodules. Histologically these were seen to be small groups of hepatoma cells which showed less vacuolation than those in in the first week and were almost encapsulated. In the other mouse the graft site was hardly visible and no liver cells were found.

Third week.—The site of grafting in both mice was less well defined than in the 1st and 2nd weeks. A group of well preserved hepatoma cells was found in one mouse and in the other a few nucleated cells which were possibly hepatoma cells were seen beside an area of reaction tissue.

Fourth week.—In one mouse three small white areas were seen through the dissecting microscope after 10 minutes' fixation in Bouin's fluid. Histologically 4 groups of well preserved hepatoma cells were found. The nuclei had prominent nucleoli and the cytoplasm was densest nearest the nuclei. The groups of cells were surrounded by fat (Fig. 2). In the other mouse a small brown graft was found macroscopically. Large masses of pigment were found histologically in cells of uncertain nature other than the hepatoma cells which formed a small group. There was no clear cut margin between the two types of cells and a few hepatoma cells were scattered in the surrounding tissue.

Fifth week.—Macroscopically a tiny white nodule and pigmented area were seen in one mouse and a less definite area in the other. Microscopically pigmented cells which were considered to be hepatoma cells were found in both grafts as well as rounded groups of hepatoma cells in the region of the white nodule.

Sixth week.—The graft site was easily visible to the naked eye in both mice as a slightly pigmented area. Several small rounded groups of vacuolated and granular hepatoma cells were seen microscopically in one mouse with some pigmented cells adjacent to them. In the second mouse no hepatoma cells were seen.

Seventh week.—No graft site could be found in either mouse even after bulk staining.

Eighth week.—In one mouse a pigmented area was seen, naked eye, after

fixation. Microscopically this was noted to be quite a large area of hepatoma cells extending in finger-like processes into the adjacent fat with a small lymphatic vessel, identified by its content of lymphocytes, at its base. The cells were vacuolated and no mitoses were seen. In the second mouse the site of grafting was not found.

Ninth week.—In one mouse a small pigmented area was seen through the dissecting microscope and a few pigmented cells of uncertain nature were found microscopically. The site of grafting was not found in the other mouse.

Tenth week.—On naked eye examination a small brown spot was seen at the site of grafting in one mouse. Under the dissecting microscope a small white nodule was noted at the edge of a speckled area. Microscopically this was seen to be composed of a group of hepatoma cells lying in a fatty network. Some of the hepatoma cells were vacuolated and granular and others pigmented. The pigment gave a positive reaction for iron. In the second mouse a similar brown speckled area was found which was composed of actively growing hepatoma cells in which many mitotic figures were seen (Fig. 3).

Eleventh week.—Two mice had just palpable grafts with pigmented and liver-like areas on naked eye examination. Microscopically they were actively growing hepatomas in which mitoses were frequent. In one there were small rounded encapsulated groups of hepatoma cells, scattered in fatty tissue around the main tumour, similar to those seen in earlier grafts. Two further mice killed at this time showed a small speckled area at the site of grafting. A few rounded groups of hepatoma cells were seen in one and small clusters of hepatoma cells scattered in a fatty network in the other.

Twelfth week.—One mouse had a palpable liver-like graft which was seen to be a trabecular hepatoma showing mitoses. In the second mouse a small pigmented area was seen macroscopically in which, microscopically, there were two small groups of hepatoma cells with flattened cells on the surface encapsulating them. A few scattered hepatoma cells were seen in surrounding fat.

Thirteenth week.—Although the grafts were not palpable they were found as small brownish areas and small white nodules were seen through the dissecting microscope in one, in which round nodules of hepatoma with mitoses were found microscopically. In the other a few pigmented cells which were probably hepatoma cells, were seen in a fatty network.

Fourteenth week.—In one mouse the graft was identified after fixation and a small rounded group of hepatoma cells and some scattered pigmented cells were found microscopically. In the other mouse a brownish speckled area showed a few pigmented cells which were undoubtedly hepatoma cells.

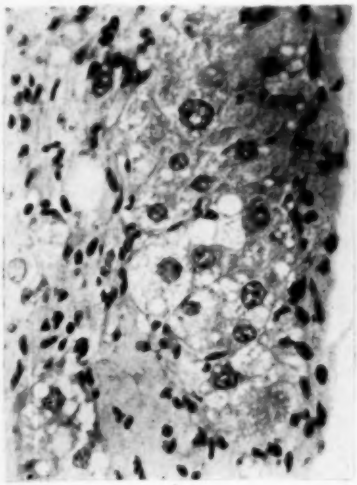
EXPLANATION OF PLATE

FIG. 1.—Vacuolated hepatoma cells lying singly and in a group one week after grafting. ×320.

FIG. 2.—Encapsulated groups of hepatoma cells lying in fatty matrix four weeks after grating. ×145.

FIG. 3.—Hepatoma graft showing mitoses ten weeks after grating. ×375.

FIG. 4.—Vena cava and liver showing the possible path of metastases from the subcutaneous graft.



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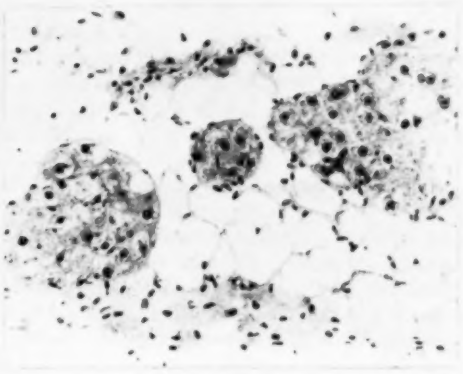

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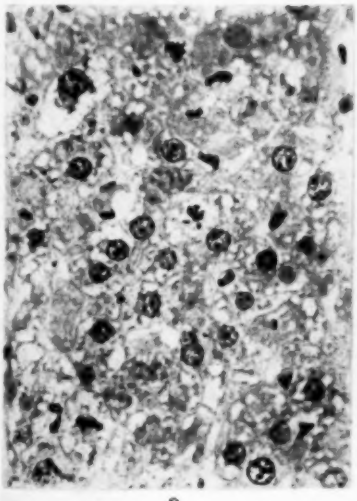

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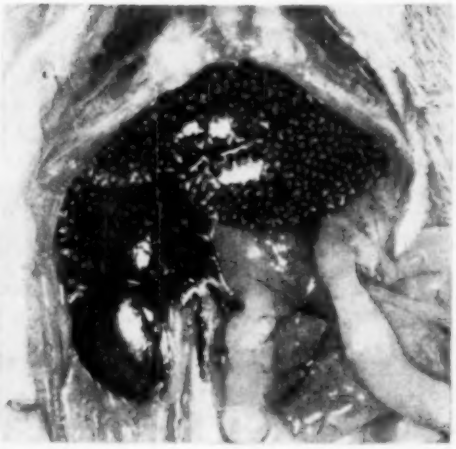

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Fifteenth week.—In one mouse the graft had been palpable for 2 weeks. It was a solid liver-like tumour with mitoses present. Small rounded groups of hepatoma cells were scattered in the fatty tissue round the main tumour. The second mouse had a tiny graft in which rounded encapsulated groups of hepatoma cells were seen adjacent to other cells which contained round granules of pigment. A third mouse, which was ill, was killed but no hepatoma cells were found although the site of grafting was found histologically.

Sixteenth week.—In two mice small pigmented grafts with white areas were seen more clearly after fixation and groups of hepatoma cells with flattened cells encapsulating them were seen histologically. No mitoses were found but some pigment was seen in cells adjacent to the graft. A third mouse which died showed similar features.

Seventeenth week.—Macroscopically, small pigmented grafts were found in both mice. After fixation many white nodules were seen in one of them in which groups of hepatoma cells containing mitoses were present. In both grafts, groups of stellate cells lay in a fatty network between the groups of hepatoma cells. Pigmented cells which may be liver cells were also seen.

Eighteenth, nineteenth, twentieth and twenty-first weeks.—No definite hepatoma cells were identified in any of the seven mice killed over this period. A small pigmented area was found in three of the mice, and a few pigmented cells were seen in four of them. Only one mouse was killed at the 21st week in order to leave sufficient mice alive.

Long term findings.—Three of the remaining five mice then had palpable tumours. They were killed 7 to 8 months after grafting when the tumours were becoming very large and the mice were 10 months old. In one of them, a back cross mouse, two small round yellow tumours were present in the liver, one in the right upper lobe and one in the left half of the middle lobe. They were deeply placed in the centre of the lobes and not standing out from the surface with large blood vessels in grooves as is usual in primary tumours of this type. They were considered to be possible metastases from the hepatoma graft as we have not found primary spontaneous tumours in previous observations in ($C_3Hf \times RIIIf$) $\times$ RIIIf mice under 14 months old nor have Pullinger and Iversen (1960) seen them in RIIIf mice under 15 months old.

We have traced a possible path of metastases to the liver by injecting white ink into the graft in a freshly killed mouse. The ink can be seen travelling along the iliolumbar vein into the inferior vena cava, thence to the hepatic veins. The liver then becomes white and mottled (Fig. 4) and sections show ink in the hepatic veins within the liver. Multiple metastases to the liver only, from subcutaneous grafts have been found in another experiment in which a lymphoblastoma was being investigated (Head and Chalmers, 1959).

Of the remaining two mice one died after 20 months and one was killed after 21 months. No sign of the graft was found.

TABLE I.—*Number of Mice with Groups of Hepatoma Cells at Site of Grafting*

5–10 weeks . . .	5/12
11–21 weeks . . .	14/25
After 21 weeks . . .	3/5

Second Series : 21 *female and* 5 *male mice*

($C_3Hf \times RIIIf) \times RIIIf$, F_1 mice aged 5 to 9 weeks were used. They were grafted subcutaneously by open operation as in the first series and 2 female mice were killed each week for 6 weeks then one at 7 and 8 weeks. The remaining 7 females and the 5 males were kept until a palpable tumour arose or the animal died. This was done in order to obtain information about the length of the latent period in female as well as male mice and to compare the appearances of the graft site in the early stage with the number of tumours in the late stage.

First week.—In both mice a small yellow pinhead-sized nodule was found at the site of grafting and a few vacuolated hepatoma cells not forming definite groups were found on microscopical examination.

Second week.—A small nodule of graft was found in both mice. Multifocal groups of growing hepatoma cells in which mitoses were present were seen microscopically in one and a few scattered hepatoma cells in the other.

Third week.—In both mice a small yellow pinhead-sized graft was found attached to the muscle of the abdominal wall. Groups of growing hepatoma cells, some vacuolated, many binucleated and others in mitoses, were seen in one mouse. In the other a few vacuolated and pigmented hepatoma cells were seen.

Fourth week.—In both mice a small yellow graft was found attached to the muscle of the abdominal wall. In addition a smaller nodule in the subcutaneous tissue was found in one. Portions of hepatoma graft showing mitoses were seen microscopically in one mouse. In the other, groups of hepatoma cells were vacuolated and the cells around them contained golden pigment.

Fifth week.—In both mice a small yellow graft was seen attached to the muscle of the abdominal wall. In one, groups of vacuolated hepatoma cells were seen lying in a fatty network. In the other, groups of hepatoma cells with a pale granular appearance were surrounded by cells containing golden pigment. No mitoses were seen.

Sixth week.—The grafts were seen as small yellow nodules in the subcutaneous tissue and one was also attached to the muscle of the abdominal wall. A few hepatoma cells, some containing golden-coloured pigment and others vacuolated, were found in one mouse and only a few possible liver cells surrounding a necrotic area in the other.

Seventh week.—The small graft was attached to the abdominal muscle and microscopically showed groups of hepatoma cells. One group stained well and showed a cell in mitosis and others showed degenerate looking cells. Some cells other than hepatoma cells contained pigment.

Eighth week.—A pigmented area smaller than any previous ones in this series was found on the muscle of the abdominal wall. A small group of poorly staining hepatoma cells similar to those seen at seven weeks was found on cutting serial sections of the graft.

At this stage the histological appearance suggested that the graft was degenerating or regressing and the remaining 7 female mice were kept to see in how many the graft had taken. Two months later, 4 months after grafting, tumours became palpable in 3 mice which were killed 7, 10 and 10 months respectively after grafting. All had hepatoma grafts which were very liver-like in appearance and had some cystic and haemorrhagic areas. Two mice were killed in extremis after 19 and 22 months and no graft was found.

The last 2 mice were killed at 26 months. Although no sign of the graft was found in either mouse, a round yellow tumour was present in the centre of the left lobe of the liver in one of them. This was seen histologically to be a hepatoma.

One of the 5 male mice had a palpable graft after 8 weeks and was killed after 7 months when it had a large, pale, fatty-looking hepatoma at the site of grafting. Another male mouse had a palpable graft after 11 months and died after 12 months. This tumour was a golden brown colour. In a third mouse the graft became palpable after 15 months. It was killed after 17 months and had a large brown liver-like graft. Microscopically, all three tumours were typical trabecular hepatomas similar to those in the female mice. Of the remaining two male mice, one died after 24 months and one was killed after 26 months and no sign of the graft was found in either.

TABLE II.—*Number of Mice with Groups of Hepatoma Cells at Site of Grafting*

2–8 weeks . . .	8/12
After 7 months . . .	3/7 female
	3/5 male

Third Series: 14 *female and* 5 *male mice*

RIIIf and (C_3Hf × RIIIf) × RIIIf 2 to 3-months old mice were used. They were grafted in the same way as the previous series by open operation. In this set it was planned to study the period after 8 weeks when the grafts in the previous series had appeared to regress. However, by the 9th week one female mouse already had a palpable graft and 4 more became palpable by the 10th week. Of the remaining 9 female mice, 2 had tumours at 12 weeks, 1 at 14 weeks, 4 at 19 weeks and 1 at 23 weeks. One female mouse died at 17 months after grafting and the site could not be identified.

Of the 5 male mice, 1 had a palpable tumour at 9 weeks, 3 at 14 weeks and 1 at 19 weeks. Two male mice in this series aged 8 months and 9½ months had small tumours in the liver. The 8-months-old mouse had had a palpable graft for 6 weeks before it was killed and the liver tumour was a small round nodule in the centre of the left lobe. The 9½-months-old mouse had had a palpable tumour for 4 months and the liver tumour was deeply situated, as in metastases, in the right upper lobe, but also projected outwards with large vessels grooving the surface, as in primary tumours. It is debatable whether this is a primary or a secondary tumour.

TABLE III.—*Number of Mice with Palpable Grafts*

After 23 weeks . .	13/14 females
	5/5 males

Fourth Series: 24 *male mice*

Twelve RIIIf and 12 (C_3Hf × RIIIf) × RIIIf, F_1 2 to 3 months old mice were grafted subcutaneously from a seventh generation transplant of the hepatoma in order to study further, the period between 5 and 10 weeks after grafting.

In this series 19 of the mice were chosen at random to be killed during this period leaving 5 until a tumour became palpable or the mouse died. This was done in order to compare the number of palpable tumours at the later stage with the histological appearance of the graft sites in the earlier stages (as in the previous

series) and to get more information about the variability in the percentage of takes from generation to generation. Twelve of the 19 mice had discrete encapsulated groups of hepatoma cells with mitoses present.

Fifth week.—Two mice were killed. In one, groups of hepatoma cells were encapsulated by flattened cells and mitoses were present. In the other no hepatoma cells were found at the site of grafting.

Sixth week.—Two mice were killed and in both mitoses were found in groups of hepatoma cells.

Seventh week.—Four mice were killed. In 2 of these, groups of hepatoma cells, some showing mitoses, were found at the graft site, which was seen as a group of several small white spots under the dissecting microscope. In a third mouse a few hepatoma cells, not definitely encapsulated, were found. In the fourth mouse no definite liver cells were seen.

Eighth week.—Four mice were killed and in 3, small pink nodules were easily found. These were seen, microscopically, to be active hepatoma grafts. In the fourth mouse the site of grafting was not identified.

Ninth week.—Four mice were killed and 2 had growing grafts. One of these was a pinhead-sized pink nodule lying in a pad of fat and microscopically showed numerous mitoses. One appeared as a group of small white nodules in the fork of a blood vessel and groups of hepatoma cells were seen near a main lymphatic vessel containing many lymphocytes. In the third mouse a few viable-looking pigmented hepatoma cells were seen lying adjacent to a small lymph node. In the fourth mouse the site of grafting was not found.

Tenth week.—Three mice were killed 2 of which had an oval brownish-coloured graft in which mitoses were common. One of these grafts was just palpable. In the third mouse no definite liver cells were found but some macrophages were found containing small homogeneous round granules of pigment.

Of the 5 remaining mice 2 had palpable tumours at 12 weeks, one at 15 weeks and one at 6 months. One died after 24 months with no sign of the graft at the site of implantation.

TABLE IV.—*Number of Mice with Groups of Hepatoma Cells*

5–10 weeks . . .	12/19
After 12 weeks . .	4/5

SUMMARY AND CONCLUSIONS

In the first series, 5 of the 12 male mice killed between 5 and 10 weeks after grafting had definite discrete groups of hepatoma cells at the site of transplantation, and 2 showed only a few scattered cells. It was noted that no hepatoma cells were found at the 7th and 9th weeks after grafting. From the 11th to the 21st week 14 of 25 mice had discrete groups of hepatoma cells and 3 only a few scattered hepatoma cells. Mitoses were first seen at the 10th week. In the later period, after 21 weeks 3 of 5 mice had palpable hepatoma grafts.

In the second series 8 of 12 female mice killed between the 2nd and 8th week had discrete groups of hepatoma cells at the site of grafting. In 3 mice only a few scattered hepatoma cells were found. It was noted that at the 8th week the graft appeared less viable than in the earlier weeks. Mitoses were seen in 4 of them at the 2nd, 3rd, 4th and 7th weeks. In the later stage 3 of 7 female mice and 3 of 5 male mice had palpable grafts.

The third series was a continuation of the second series, in order to study the grafts after 8 weeks. The latent period before the earliest graft became palpable was 9 weeks. Thirteen of the 14 female mice and all of the 5 male mice had palpable grafts by the 23rd week. The portions of tissue implanted in this series were larger than in the previous sets and this was considered a possible reason for the higher percentage of takes.

The fourth series was grafted in order to repeat the period from 5 to 10 weeks in male mice. In the early stage from the 5th to the 10th week 12 of 19 mice had definite discrete groups of hepatoma cells and 2 only a few scattered hepatoma cells at the site of grafting. Mitoses were first seen at the 5th week. In the later stage 4 of 5 mice had palpable grafts.

The appearance of mitoses by the 5th week in the fourth series and by the 10th week in the first series indicates that the graft had established itself in a certain proportion of the mice during this time. We originally thought that only the well encapsulated groups of hepatoma cells survived and grew into palpable grafts. However, in view of the number of tumours in the later stage it is probable that some of the scattered hepatoma cells, identified in the early stages, can become active later and may account for the much longer latent period in some mice of the same set.

There are no significant differences between the growth in female and male mice in the generations studied in these experiments.

Some grafts from different tumours in the same generation show a higher percentage of take which may be due to age, or composition of the tumour selected for grafting. In the third series the portions of tissue grafted were larger than in the first two series which may account for more takes in this group.

The latent period, which was taken as the time from grafting to detection of a palpable tumour by weekly examination, varied from 2 to 15 months. In the first generation it was 5 to 14 months but was reduced to 2 to 3 months in the 5th, 6th and 8th generations.

In the later stage of the experiment, mice without palpable tumours were not killed till 20 to 26 months after grafting. One died after 17 months in the third series, and one was killed in extremis in the second series after 19 months.

Possible metastases were found in the liver in 3 mice with large grafts.

Our findings agree with Andervont and Dunn's (1952) that not all grafts grow in early transplant generations although hosts are genetically comparable and the tumours are potentially malignant.

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REFERENCES

AGNEW, L. R. C. AND GARDNER, W. U.—(1952) *Cancer Res.*, **12**, 757.
ANDERVONT, H. B.—(1950) *J. nat. Cancer Inst.*, **11**, 581.
Idem AND DUNN, T. B.—(1952) *Ibid.*, **13**, 455.
BURNS, E. L. AND SHENKEN, J. R.—(1940) *Amer. J. Cancer*, **39**, 25.
HEAD, M. A. AND CHALMERS, J. G.—(1959) *Rep. Brit. Emp. Cancer Campgn.*, **37**, 434.
Idem AND LAIRD, H. M.—(1957) *Ibid.*, **35**, 302.—(1958) *Ibid.*, **36**, 405.—(1959) *Ibid.*, **37**, 434.
LAIRD, H. M.—(1955) *Ibid.*, **33**, 282.
Idem AND HEAD, M. A.—(1956) *Ibid.*, **34**, 282.
PULLINGER, B. D. AND IVERSEN, S.—(1960) *Brit. J. Cancer*, **14**, 267.

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CORRELATIONS OF HISTOLOGICAL AND BIOCHEMICAL CHANGES IN THE LIVERS OF RATS DURING PROLONGED ADMINISTRATION OF ACETYLAMINOFLUORENE

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WILSON, De Eds and Cox (1941) showed that the administration of 2-acetylaminofluorene (AAF) to rats causes epithelial hyperplasia in a number of organs. Subsequent investigations by Bielschowsky (1944), Matsus (1954) and Farber (1956) have demonstrated that some of these hyperplasias are in fact malignant hepatomas.

When rats are fed hepatic carcinogen such as the azo dyes (Miller and Miller, 1955) and thioacetamide (Grant and Rees, 1957) the intracellular composition and the enzyme activity of the liver cell may be altered. Grant and Rees (1957) found that thioacetamide feeding causes cholangiocarcinoma along with a decline in the phospholipid synthesis, a rapid decrease in the mitochondrial fraction and the "ageing" phenomenon of mitochondria in liver cells. Rats fed on a diet containing dimethylaminoazobenzene (DAB) and supplemented with B vitamins do not show these changes although ultimately they develop hepatocarcinomas.

According to Laird and Miller (1953) and Rutman, Cantarrow and Paschkis (1954) AAF alters the intracellular composition of the rat liver. The purpose of this paper is to investigate phospholipid and ribonucleic acid (RNA) synthesis, the "ageing" phenomenon of mitochondria and the levels of respiratory enzymes in the liver undergoing AAF carcinogenesis and to correlate these measurements with the histological picture.

METHODS

Animals.—150 Stock male albino Wistar rats, weighing 90–200 g. were used in this study, and each animal was weighed at weekly intervals.

Diet.—2-Acetylaminofluorene was added to powdered M.R.C. rat cubes to maintain a dosage of 0·5 mg. per cent. Animals were given food and water *ad libitum*.

Histology.—All organs were examined microscopically. The wet weights of the liver were measured and expressed as percentage of body weight. Histological examinations were confined to the liver, slices of which were fixed in Carnoy's fluid. Sections, 5 μ. thick, were stained with Ehrlich's acid haematoxylin and eosin, periodic acid-Schiff technique (PAS), pyronin methyl green, Feulgen's technique, van Gieson, Best's carmine, muci-carmine, and with Gomori reticulin stains.

Biochemical and Chemical Determinations

Homogenates and mitochondria were prepared in 0·25 M sucrose (Schneider, 1948).

For spectrophotometric determinations homogenates and mitochondrial suspensions were frozen and thawed at least four times before use. This procedure ensured maximum enzyme activity (Kielley, 1957).

Malic dehydrogenase and glutamic dehydrogenase were determined by the method of Christie and Judah (1953).

*Iso*citric dehydrogenase was determined by the method of Hogeboom and Schneider (1950).

The RNA and phospholipid content of the livers was determined by the method of Davidson, Frazer and Hutchinson (1951).

Synthetic reactions

The rate of ^{32}P incorporation was determined *in vivo* following the intraperitoneal injection of inorganic ^{32}P to supply 100 μC/kg. body weight. The animals were killed after 4 hours. The phospholipid fractions of the liver were estimated by the method of Kennedy (1953). Radioactivity was determined in films of zero thickness on metal planchettes using an end window counter. The *in vivo* synthesis of RNA was measured by a modification of the method of Davidson *et al.* (1951). Inorganic ^{32}P was administered intraperitoneally at 100 μC/kg. body weight. The rats were killed after 4 hours and the liver perfused with 20 ml. 0·25 M sucrose. Portions of the liver were homogenised, made to 10 per cent w/v suspensions and the protein precipitated with trichloroacetic acid (final concentration 5 per cent w/v). The supernatant was estimated for RNA by the method of Davidson *et al.* (1951). Radioactivity was determined in a liquid-counter of conventional design.

RESULTS

Pathological changes

2-Acetylaminofluorene has a high specificity for the liver, although it is frequently carcinogenic for several other organs and tissues (Wilson *et al.*, 1941). None of our animals developed tumours of any organ other than the liver during the fifty-three week period of observation. After 1 month of feeding, the histological changes are almost all confined to the parenchymal cells of the liver, the periportal region being the most severely affected. Degenerate and atrophic liver cells are present. The earliest change observed is a loss in the staining property of the nucleoli. Occasionally central hepatic cells increase in size and their nuclei become enlarged. Many mitoses are seen at this stage among the healthier central hepatic cells. There is no bile duct proliferation. During the fourth month, as the result of progressive degeneration and death of a large number of liver cells, there is collapse of parenchymal tissue and the liver becomes granular. The resultant condensation of the hepatic reticulin together with the increased fibrous tissue around portal tracts and slight bile duct proliferation leads to the formation of bands of cellular connective tissue uniting most portal tracts, whilst elsewhere regeneration of hyperplastic nodules are fairly prominent. However, during the fifth month there is more pronounced regeneration of liver cells and

some resolution of the fibrous tissue takes place. It is at this stage that a new glandular pattern appears (Stewart and Snell, 1957) in the proliferating bile ducts which become dilated or sometimes cystic giving the picture which Opie (1944) has termed cholangiofibrosis. The picture remains substantially unaltered up to seven months of feeding. The dry weight of the livers remain unchanged despite a progressive hyperplasia of hepatic cells and the onset in some rats of malignant change in the liver. By the tenth month practically all the animals have developed hepatocarcinomas. No tumours of bile duct were identified.

Chemical and biochemical investigations

Rats were killed at regular intervals, liver homogenates were prepared and their nitrogen, ribonucleic acid and phospholipid content were determined, over the 52 weeks of feeding (Table I). The total nitrogen and RNA content fell

TABLE I.—*The Effect of Acetylaminofluorene Feeding on the Total Nitrogen, RNA and Phospholipid Content of the Liver*

All the results are calculated on 100 mg. wet weight liver and are the means and range from five animals at each time interval

Duration of feeding AAF (weeks)	mg. N	µg. RNA. P	µg. phospholipid P
0	3·19 (3·07–3·13)	76·5 (73–81·5)	129 (110–137)
12	3·19 (3·1–3·3)	77·1 (71·5–81·5)	132 (121–142)
28	3·17 (3·2–3·15)	67·5 (67·5–73)	125 (121–130)
32	2·95 (2·85–3·05)	53·2 (50·58)	129 (115–142)
36	2·85 (2·8–2·85)	58·5 (50·61)	135 (130–140)

progressively whereas the phospholipid content of the liver remained unaltered. The liver homogenates were fractionated by differential centrifugation and the nitrogen content of the various fractions was assessed (Table II). A progressive

TABLE II.—*Changes in Intracellular Distribution of Nitrogen in the Liver following the Feeding of Acetylaminofluorene*

The results are expressed as mg. N/g. wet weight of liver.

Duration of AAF feeding (in weeks)	Animal number	Whole liver	Nuclei	Mitochondrial fraction	Supernatant
0	1	32·0	5·6	9·3	13·3
	2	32·0	5·8	9·3	12·9
12	1	30·1	8·1	8·0	12·1
	2	30·0	7·9	8·2	12·2
35	1	28·5	8·2	6·4	12·8
	2	28·0	8·2	6·7	12·6
52	1	10·0	10·0	5·1	12·0
	2	27·8	9·8	5·3	12·2

fall in mitochondrial nitrogen occured which was well marked by 35 weeks in contrast to the rise met with in that of the nuclear fraction. The supernatant fraction remained constant throughout the whole experiment.

The glutamic, malic and *iso*citric dehydrogenase content of the liver homogenates were also determined (Table III). The activities of *isocitric* and malic

TABLE III.—*The Level of* Isocitric, *Malic and Glutamic Dehydrogenase in Livers of Animals Feed Acetylaminofluorene*

Results for dehydrogenases as μmoles DPN reduced/g. wet wt./hr. and are the means and range from five animals at each time interval.

Duration of feeding AAF (weeks)	Dehydrogenases		
	Malic	Isocitric	Glutamic
0	1040 (1020–1060)	610 (590–620)	279 (258–300)
12	1040 (1030–1070)	555 (520–590)	275 (260–290)
28	1015 (1030–980)	610 (520–630)	—
32	725 (710–750)	445 (430–460)	181 (182–210)
52	560 (510–580)	435 (420–450)	214 (208–220)

dehydrogenase, fell both in relation to the wet weight of the liver and its nitrogen content but with glutamic dehydrogenase the fall in activity was much smaller.

Dry weight determinations on the livers of control and treated animals were made throughout the feeding experiment. The values for the livers of treated animals were the same as those of the controls.

The oxidation of the substrates (L-malate, L-glutamate and α-oxoglutarate by mitochondrial preparations from the livers of AAF-treated rats was determined. Up to 25 weeks of feeding the levels of oxidation in terms of mitochondrial nitrogen remained much the same as that of control animals. The mitochondrial preparations in 0·25 M sucrose on storage at 0° for 6 hours showed no decline in oxidative activity.

During the course of feeding AAF the incorporation of ^{32}P into the phospholipid and RNA of the liver was followed. In all these experiments the serum inorganic hosphate was determined and found to be the same for both control and treated rat in any given experiment. Thus the isotope dilution was identical in both groups. It may be seen from Table IV that no decline in ^{32}P incorporation into

TABLE IV.—*The Incorporation of ^{32}P as Inorganic Phosphate into the Ribonucleic Acid of Phospholipids of the Livers of Both Control and Treated Rats*

The results are from individual animals.

Duration of feeding AAF (weeks)	Percentage decrease or increase in specific activity compared to controls	
	Phospholipids	Ribonucleic acid
19	+5	+2
	+1	+4
34	−8	+1
	−10	−4
52	−20	−2
	−18	0

the phospholipids occurred until after 35 weeks, in fact not until the onset of the hepatomas. In no case did the incorporation of ^{32}P into the liver RNA of the treated animals differ from that of their controls.

DISCUSSION

During the exposure of rats to hepatocarcinogens a redistribution occurs of the protein and RNA in the liver cell. There is generally, an increase of these substances in the nuclear fraction and a fall in the cytoplasmic fraction, in par-

ticular that of the mitochondria (Miller, 1955 ; Grant and Rees, 1957). Administration of AAF likewise leads to an increase in the protein and RNA content of the nuclear fraction and a fall in the mitochondrial fraction. These findings agree with those of Rutman, Cantarrow and Pashkis (1954). With AAF, however, unlike dimethylaminoazobenzene and thioacetamide carcinogenesis there are alterations in the overall composition of the liver. Our animals showed a progressive fall in the liver nitrogen and RNA but there was no change in the phospholipid content.

Measurements of the respiratory enzymes in the liver support the conclusion that there is a fall in the mitochondrial population rather than alterations in the enzymic content of the mitochondria. There is a fall in the glutamic, *iso*citric and malic dehydrogenase content of both liver homogenates and mitochondrial suspensions. However, if the results of the mitochondrial experiments are expressed in terms of mitochondrial nitrogen the values are the same as those for preparations from the livers of control animals.

Grant and Rees (1957) investigating dimethylaminoazobenzene and thioacetamide carcinogenesis concluded that a cholangiocarcinoma is most likely to develop when the liver shows damage to its parenchymal cells, proliferation of bile duct, tissue, a drop in the mitochondrial fraction and the phenomenon of " ageing " of mitochondria. During this time there is a gradual fall in the phospholipid synthesis and finally a tumour develops. In contrast, hepatocarcinoma results if this precancerous process is slowed down. Parenchymal cell damage though qualitatively similar to that seen in the more rapid cases leads to the death of fewer cells. In such animals the mitochondrial population is not depleted, mitochondria do not exhibit " ageing " and phospholipid synthesis is unaltered until the onset of the hepatoma

The criteria were applied to the problem of acetylaminofluorene carcinogenesis. Little or no bile duct cell proliferation and little liver cell death is seen until sixteen weeks of feeding AAF. Phospholipid synthesis is unaltered until the onset of neoplasia and mitochondrial preparations do not exhibit the " ageing " phenomenon. There is, however, a fall in the mitochondrial fraction but this is not as great as was found in thioacetamide carcinogenesis (Grant and Rees, 1957).

All such events would seem to point to the development of parenchymal cell tumours rather than bile duct tumours, in rats fed AAF. Histological investigation has shown that this actually is the case. All of our liver tumours have been undoubted hepatocarcinomas. We have not encountered a single cholangiocarcinoma.

SUMMARY

1. The histological and biochemical events occurring in acetylaminofluorene hepatocarcinogenesis in rats have been followed.

2. As with other hepatocarcinogens there are increases in nuclear material and a fall in the cytoplasmic fraction. There is a progressive fall in the number of mitochondria but enzymatically they appear to be unaltered, they do not exhibit the phenomenon of " ageing ".

3. There is no change in phospholipid and ribonucleic acid synthesis *in vivo* during the tumour induction period.

4. It is considered that these events should precede the production of hepatocarcinoma which is the tumour type produced in these experiments.

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REFERENCES

BIELSCHOWSKY, F.—(1944) *Brit. J. exp. Path.*, **25**, 1.
CHRISTIE, G. S. AND JUDAH, J. D.—(1953) *Proc. Roy. Soc.* B, **141**, 420.
DAVIDSON, J. N., FRAZER, S. C. AND HUTCHINSON, W. G.—(1951) *Biochem. J.*, **49**, 311.
FARBER, E.—(1956) *Cancer Res.*, **16**, 142.
GRANT, H. C. AND REES, K. R.—(1957) *Proc. Roy. Soc.* B, **148**, 117.
HOGEBOOM, G. H. AND SCHNEIDER, W. C.—(1950) *J. biol. Chem.*, **186**, 417.
KENNEDY, E. P.—(1953) *Ibid.*, **201**, 399.
KIELLEY, R. K.—(1957) *Biochim. biophys. Acta*, **23**, 447.
LAIRD, A. K. AND MILLER, E. C.—(1953) *Cancer Res.*, **13**, 464.
MATSUS, H.—(1954) *Med. J. Osaka Univ.*, **5**, 763.
MILLER, E. C. AND MILLER, J. A.—(1955) *J. nat. Cancer Inst.*, **15**, 1571.
OPIE, E. L.—(1944) *J. exp. Med.*, **80**, 231.
RUTMAN, R. J., CANTBARROW, A. AND PASCHKIS, K. E.—(1954) *Cancer Res.*, **14**, 111.
SCHNEIDER, W. C.—(1948) *J. biol. Chem.*, **176**, 259.
STEWART, H. L. AND SNELL, K. C.—(1957) *Acta Un. int. Cancr.*, **13**, 770.
WILSON, R. H., DE EDS, F. AND COX, A. J.—(1941) *Cancer Res.*, **1**, 595.

A THIOL PEPTIDE RELATED TO GLUTATHIONE IN RATS INJECTED WITH HEPATOCARCINOGENS

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In a preceding paper (Neish and Rylett, 1960) it was noted that an unidentified ninhydrin-positive spot X appeared in electropherograms of phosphate buffer extracts of livers of male rats which had been injected intraperitoneally with powerful hepatocarcinogens. Spot X was observed in the early stages of action of the hepatocarcinogens when the livers contained high levels of phosphoethanolamine. Another unidentified spot Y sometimes accompanied X in these electropherograms.

Intraperitoneal injections of weak hepatocarcinogens or of non-hepatocarcinogens (e g. 2-methyl-4-dimethylaminoazobenzene and carbon tetrachloride) failed to promote the appearance of X or Y in rat liver. Traces of X but never of Y were sometimes found in extracts of livers of normal rats, both male and female.

Spots X and Y migrated towards the anode on Whatman No. 1 paper saturated with barbitone buffer (ρH 8·6 ; 360 volts ; 3 hours) and, with respect to easily identifiable spots due to phosphoethanolamine (PE), glutathione (GSH), glutamic acid (glu) and aspartic acid (asp), they occupied the following positions on the electropherograms :

X, PE + GSH, glu, asp and Y,

with Y lying nearest to the anode.

It was of interest to identify X and Y which may be abnormal metabolic products of liver or normal metabolic products in abnormally large amounts and also to determine whether X and Y can be detected in rat liver under other experimental conditions and in the livers and organs of other species.

Substance X and appreciable amounts of Y were found in extracts of the spleens of rats injected with 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB). They were absent in extracts of the kidneys of these rats and they could not be detected in spleen or kidneys of normal rats.

A spot corresponding to X was found in the livers of rats bearing transplantable or induced tumours and it also appeared frequently in extracts of the tumours themselves. Although spot Y was never detected in extracts of livers of tumour-bearing rats, a substance with the same mobility was seen regularly in the tumour extracts. Sometimes in electropherograms of extracts of livers from tumour rats but never in normal rat liver extracts there appeared a ninhydrin-positive spot Z which had a lower mobility than spot X under the standard conditions of electrophoresis. A spot similar to Z occurred in extracts of all the tumours examined.

Electropherograms of extracts of livers of normal male guinea-pigs contained appreciable amounts of substance X and also materials corresponding to Y and Z.

Although guinea-pig liver substance X seems to be identical in every respect with the rat liver substance X induced by a powerful hepatocarcinogen such as 3′-MeDAB, intraperitoneal injection of this carcinogen into a male guinea-pig failed to cause any noticeable elevation in the level of substance X.

Evidence has been obtained that spot X from hepatocarcinogen-treated rat liver, from the livers of rats bearing tumours and from normal guinea-pig liver is a thiol peptide closely related to but not identical with glutathione. Very few studies of spot Y have been made but it seems that this substance also may be a thiol peptide.

Tumour Z spot appears to be a protein or polypeptide particularly rich in glutamic acid, aspartic acid, glycine and cysteic acid. Chromatography of hydrolysates of Z shows that it has approximately the same qualitative and quantitative amino acid composition whether it is derived from slow or rapidly growing transplantable tumours or from chemically-induced tumours. On the other hand, Z spots observed in the electrophoresis of extracts of tissues other than tumours seem to be due to cysteine or cystine.

Some preliminary observations on the nature of spots X, Y and Z form the subject of the present paper.

EXPERIMENTAL

A description of experimental procedures has already been given (Neish and Rylett, 1960). Slow growing transplantable rat tumours originally induced with 9, 10-dimethyl-1, 2-benzanthracene (DMBA) and some rapidly growing long-established transplantable Rd/3 rat sarcomas were examined. Several chemically-induced (3, 4-benzopyrene ; BP) rat tumours were available for study.

The tumour-bearing animals were killed with ether and the livers perfused with chilled normal saline. Tumours, livers and other organs were stored at −15° C. Phosphate buffer (pH 7·2) extracts of fresh tumour tissue were prepared as previously described for liver. In a similar manner, extracts were prepared from the perfused liver and other organs of guinea-pigs.

In agreement with Sauberlich and Baumann (1951) it was found that all the tumours had a low dry weight percentage ranging from about 12 to 16 per cent of the tissue wet weight. Livers of tumour-bearing rats had a dry weight content of about 28 per cent, the same as for normal rat liver.

RESULTS

1. *Nature of substance X*

(a) *Detection of substance X by paper electrophoresis.*—Fig. 1 (A and B) illustrates the occurrence of spot X in extracts of male rat livers 4 days after intraperitoneal injection of the hepatocarcinogen, 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB ; 16·5 mg. in 0·6 ml. of arachis oil per 100 g. body weight). Spot X is absent or barely detectable in comparable extracts of the livers of normal male rats (Fig. 1, C and D) which received arachis oil only. For comparison, an electropherogram of an extract of normal male guinea-pig liver in which spot X is prominent is shown in Fig. 1 E. In this separation spots Y and Z can also be seen.

(b) *Two dimensional chromatography of substance X and its hydrolysis products.*—Paper sections corresponding to the position of the ninhydrin-positive

spot X were cut from 3 separate parallel electropherograms (each obtained from 0·01 ml. of liver homogenate supernatant) and the strips were eluted with deionised water. The aqueous extracts were freeze-dried and the residues were subjected directly or after acid hydrolysis (0·1 ml. of 6 N HCl in sealed tubes at 110° C. for 24 hours followed by freeze-drying) to two dimensional chromatography in Bowden's system (Bowden, 1959) using the modified Datta frame for multiple separations as previously described (Neish and Rylett, 1960).

The hydrolysed and unhydrolysed residues were extracted with small volumes of deionised water and these extracts, which contained undissolved fine white crystalline material (evidently barbitone buffer components and their hydrolysis products) were applied at the origin of the paper chromatograms. Apparently, the suspended material did not interfere markedly with the separation of amino acids. However, allowance had to be made for the occurrence of several ninhydrin-positive artifacts which were detected as follows. Chromatography of hydrolysed and unhydrolysed residues obtained from strips of blank electropherograms (taken from the anode side of the paper at regions where X and GSH are usually located) revealed that hydrolysed residues contain a substance which gives a small pale purple ninhydrin-positive spot with R_f values (phenol, 0·29 ; butanol, 0·15) approximately the same as those given by glutamic acid, while unhydrolysed material gives an orange-yellow ninhydrin positive spot with R_f 0·30 in phenol and 0·38 in butanol. Unhydrolysed barbitone residues also yielded a yellow diffuse spot which was seen after the phenol run (R_f 0·69). This coloured spot disappeared after the butanol run and no ninhydrin-positive material corresponding to it could be detected in the chromatograms.

The yellow ninhydrin-positive spot (N) can be seen in a chromatogram of unhydrolysed extract of spot X (Fig. 2A). The very small pale purple barbitone artifact is obscured by the stronger glutamic spot which occurs in hydrolysates of X or GSH.

In Fig. 2 are shown chromatograms of unhydrolysed X substance (A) and of hydrolysed X substance (B) obtained from electropherograms of 3′-MeDAB-treated rat liver. The products of hydrolysis of X substance from normal guinea-pig liver are seen in chromatogram D. Unhydrolysed guinea-pig liver X substance gave a chromatogram similar to Fig. 2A. Fig. 2C shows the chromatographic behaviour of the hydrolysate obtained from an authentic sample of GSH which had been processed electrophoretically exactly as described for substance X. Unhydrolysed GSH gave a chromatogram identical with Fig. 2A.

Unhydrolysed X or GSH each gave a single pale purple spot with about the same R_f values. These substances did not contain free thiol groups since the addition of *N*-ethylmaleimide (see later) to extracts containing them had no effect on their two dimensional coordinates. The substances had about the same R_f values as the sulphonic acid derivative of GSH (GSO_3H) a specimen of which was prepared by performic acid oxidation of GSH according to Kermack and Matheson (1957). Synthetic GSO_3H moves towards the anode with greater mobility than GSH. Evidently GSH and substance X undergo oxidation to derivatives similar to GSO_3H during their removal from the electropherograms.

Fig. 2B, 2C and 2D each show three ninhydrin-positive spots due to glycine (reddish) glutamic acid (strong purple) and cysteic acid (pale purple). The latter spot has a low R_f value in phenol and, in the butanol direction its R_f value is a little greater than that of synthetic GSO_3H, of unhydrolysed X (Fig. 2A) or of

GSH processed electrophoretically. Its R_f values are identical with those given by authentic cysteic acid (and incidentally with those of homocysteic acid).

Thus it seems that substance X from hepatocarcinogen-treated rat liver or from normal guinea-pig liver has the same qualitative but apparently not the same quantitative amino acid composition as glutathione. X is not identical with authentic glutathione because the latter (applied to electropherograms as a solution in pH 7·2 phosphate buffer) migrates ahead of X as a single spot to the same position as spot P (Fig. 1) occupied by phosphoethanolamine and GSH derived from liver extracts.

(c) *Presence of a free thiol group in substance X.*—The following examples illustrate the use of *N*-ethylmaleimide (NEM) in determining the thiol nature of substance X derived from fresh phosphate extracts of a number of tissues and also serve to demonstrate the occurrence of other unknown ninhydrin-positive materials Y and Z mentioned in the introduction.

A normal male guinea-pig (1000 g.) received an intraperitoneal injection of 6 ml. of arachis oil and another male guinea-pig (965 g.) was injected intraperitoneally with a solution of 3′-MeDAB (159 mg.) in arachis oil (6 ml.) The animals were killed 4 days after injection. Thus the conditions were comparable with those obtaining in the rat experiment illustrated by Fig. 1.

In Fig. 3 are shown electropherograms of 0·01 ml. aliquots of phosphate extracts of the normal (A) and 3′-MeDAB-injected (C) guinea-pig livers and of 0·01 ml. aliquots of 1 ml. portions of the same phosphate extracts to which a few crystals of NEM had been added just before electrophoresis (Fig. 3, B and D respectively).

Spot X is prominent in extracts of both normal and hepatocarcinogen-treated livers and it is evident that the carcinogen has failed to produce any major increase in the amount of X in guinea-pig liver such as was found in the case of rat liver (Fig. 1). Addition of NEM to phosphate extracts of guinea-pig liver led to the disappearance of X, a diminution in the intensity of spot P (PE + GSH) and to the appearance of a new spot Z′. It will be noted that the liver electropherograms already contain a composite spot which is termed Z in approximately the region at which Z′ appears. Following the addition of NEM (Fig. 3B) spot Z (or part of it) disappears and is replaced by a spot of lower mobility marked Z + NEM. There is reason to believe that spot Z may be due to cysteine + cystine and that Z + NEM represents the reaction product of NEM with cysteine. Without discussing the behaviour of all of these spots at present it may be remarked that spot Z′ appears to consist of the reaction products of NEM with the unknown substance X and with the GSH portion of the composite spot P which contains PE, GSH and possibly also some oxidised glutathione, GSSG.

In Fig. 4 a substance corresponding to X is seen in the electropherogram A of a fresh extract of the liver of a rat bearing a transplantable Rd/3 sarcoma and also in the electropherogram C of an extract of the tumour itself. Addition of NEM to these extracts resulted in the disappearance of spot X, diminution in the intensity of spot P and the appearance of a new spot Z′. Note that a spot Z is already present in the tumour extract (Fig 4C).

Precisely the same effects were observed in electropherograms of fresh extracts of hepatocarcinogen-treated rat liver to which NEM had been added just before electrophoresis namely spot X disappeared, spot P diminished in intensity and a new ninhydrin-positive spot with mobility Z′ appeared.

UMI

EXPLANATION OF PLATES

FIG. 1.—Electropherograms of phosphate extracts of:

A and B—rat liver 4 days after injection of 3′-MeDAB in arachis oil.
C and D—rat liver 4 days after injection of arachis oil.
E—normal guinea-pig liver.
X, Y and Z—explained in text.
P = spot due to phosphoethanolamine + glutathione.
GLU = glutamic acid.
ASP = aspartic acid.

Note: low level of aspartic acid in guinea-pig liver as compared with rat liver.

FIG. 2.—Two-dimensional chromatogram of:

A—unhydrolysed X spot
B—hydrolysed X spot
} from phosphate extract of liver of rat injected with 3′-MeDAB.

C—hydrolysate of glutathione processed electrophoretically.
D—hydrolysed X spot from normal guinea-pig liver.
N = barbitone artifact.
CYS = cysteic acid.

Vertical arrows show direction of phenol–water (4 : 1) run.
Horizontal arrows show direction of butanol-methyl ethyl ketone-dicyclohexylamine–water (100 : 100 : 20 : 47) run.

FIG. 3.—Electropherograms of phosphate extracts of:

A—guinea-pig liver, 4 days after arachis oil injection.
B—as for A, with *N*-ethylmaleimide added.
C—guinea-pig liver, 4 days after injection of 3′-MeDAB in arachis oil.
D—as for C with *N*-ethyl maleimide added.

FIG. 4.—Electropherograms of phosphate extracts of:

A—liver (8·4 g.) of female rat (total body weight = 171 g.) bearing Rd/3 sarcoma (23·2 g.)
B—as for A with NEM added.
C—Rd/3 sarcoma.
D—as for C with NEM added.

Note: high level of aspartic acid in liver as compared with low level in tumour.

FIG. 5.—Electropherograms of:

GSH—glutathione.
GSH + NEM = glutathione with *N*-ethylmaleimide added.
GSA—*S*-acetylglutathione.
GSA + NEM—*S*-acetylglutathione with *N*-ethylmaleimide added.

FIG. 6.—A. Electropherograms of:

GSH—glutathione.
GSH + NEM—glutathione + *N*-ethylmaleimide.
GSH + UV
GSH + NSC
} see text.

Two-dimensional chromatograms of:

B—unhydrolysed
C—hydrolysed
} spot "X" from GSH + UV.

D—unhydrolysed
E—hydrolysed
} spot "X" from GSH + NSC.

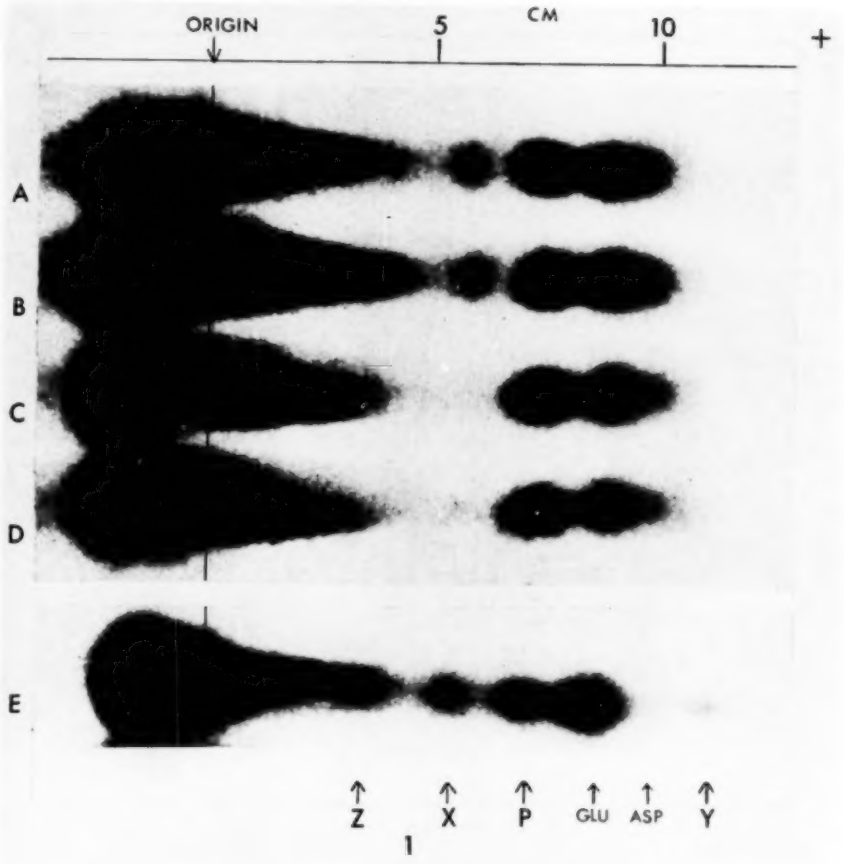
Note traces of X and cysteic acid. N = barbitone artefact.
Vertical and horizontal arrows indicate direction of flow of phenol and butanol solvents respectively.

FIG. 7.—A. Electropherograms of phosphate buffer extract of a BP tumour (1st transplant generation) before (BP) and after (BP + NEM) addition of NEM. Note failure of X to respond to NEM addition. Phosphate buffer extract several weeks old.
Two-dimensional chromatograms of:

B—hydrolysate of Z spot (see Fig. 7A).
Note distortion of glutamic acid and aspartic acid spots, due to barbitone salts.
C—Reference chromatogram. 1 mg. of each amino acid in 5 ml. of phosphate buffer (pH 7·2). 0·01 ml. of mixture applied at origin.

ALA	α-alanine	GLY	glycine	SER	serine
ARG	arginine	HIS	histidine	TAU	taurine
ASP	aspartic acid	LEU	leucine	VAL	valine
CYS	cysteic acid	LYS	lysine		
GLU	glutamic acid	PRO	proline		

Vertical and horizontal arrows indicate direction of flow of phenol and butanol solvents respectively.



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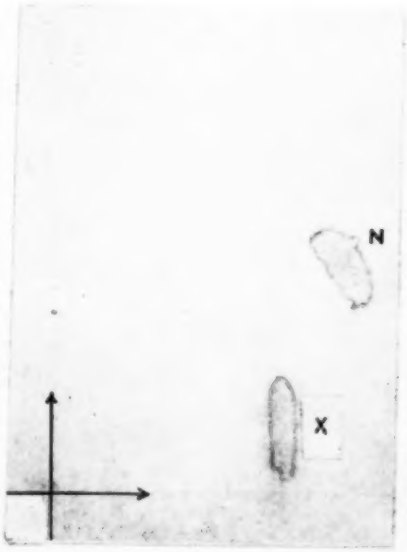

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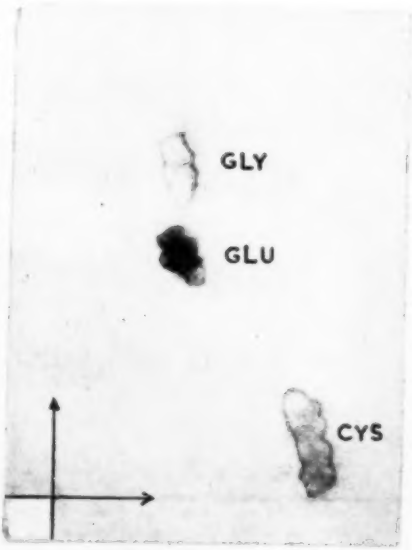

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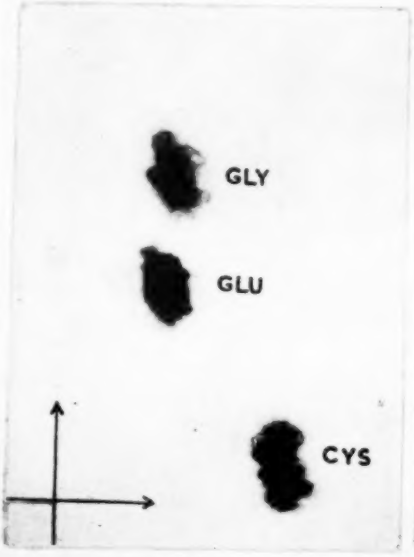

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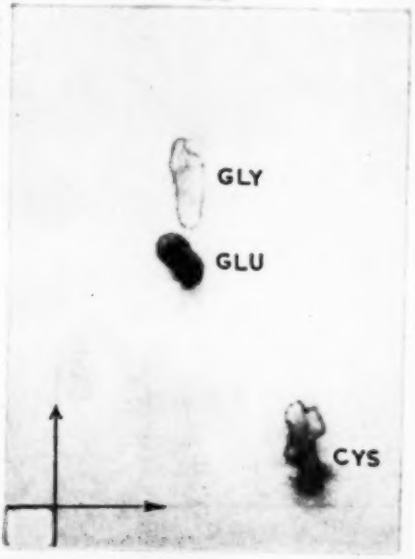

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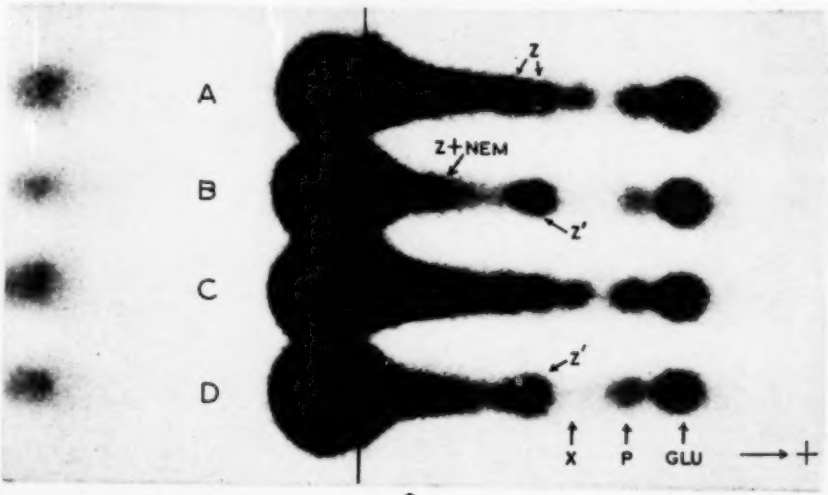

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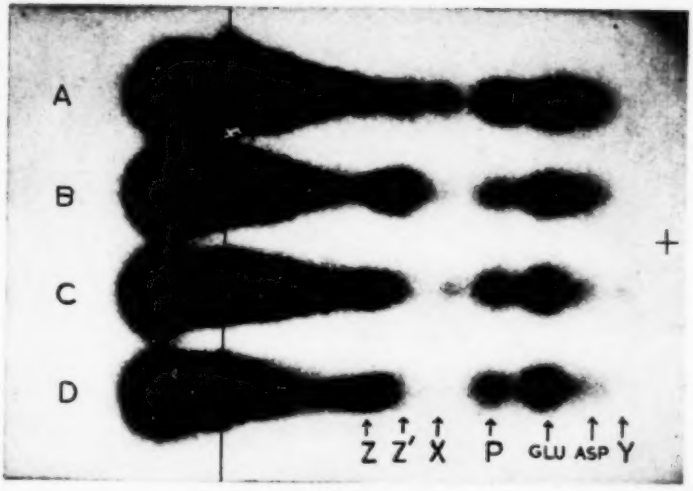

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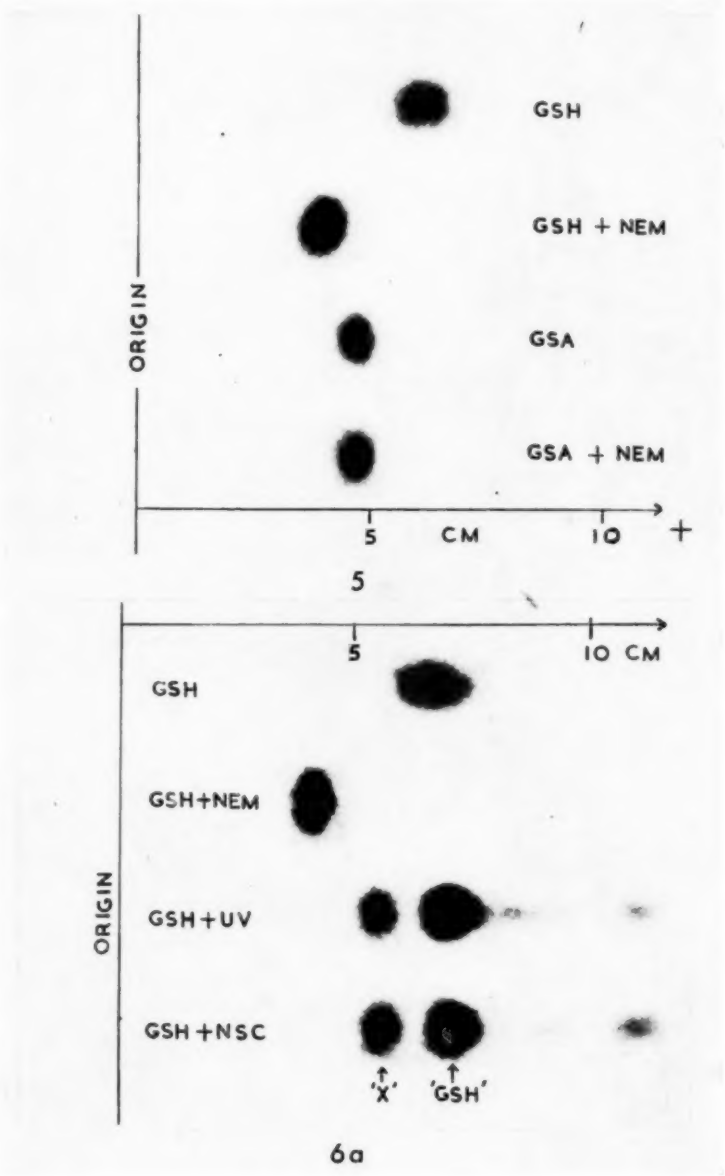
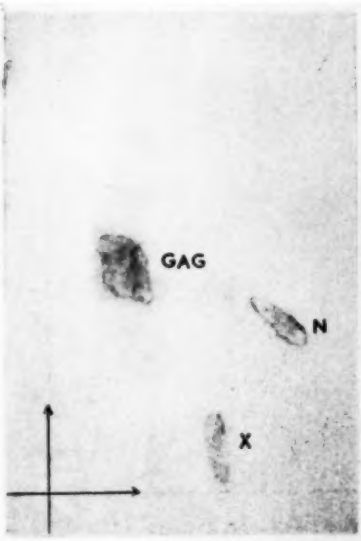
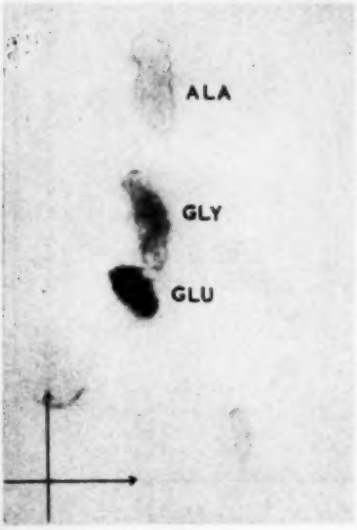

GSH
GSH + NEM
GSA
GSA + NEM
ORIGIN
5
CM
10
+
5
5
10 CM
GSH
GSH+NEM
GSH+UV
GSH+NSC
ORIGIN
'X'
'GSH'

5

6a



6b



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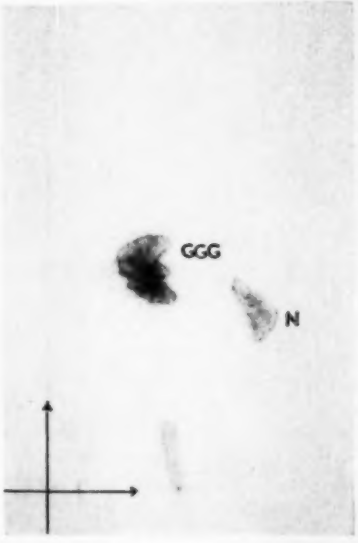

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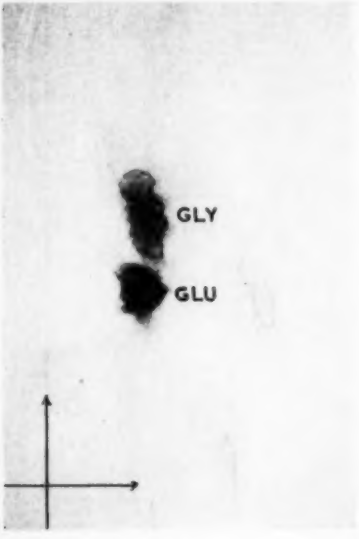
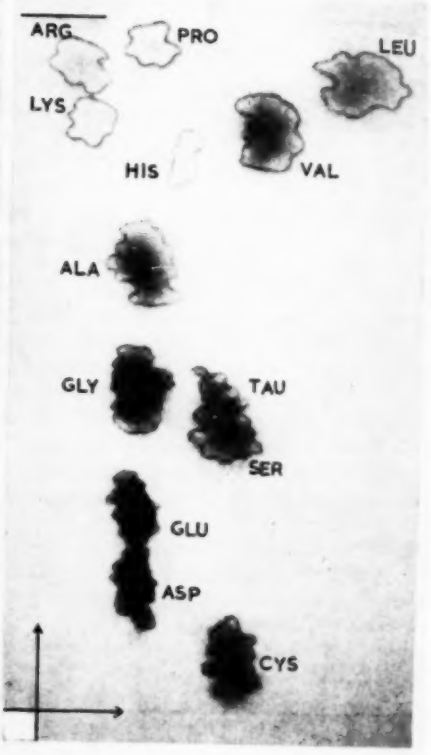


6e

BP
BP+NEM
ORIGIN
Z
X
P
GLU
ASP
Y
+

7a

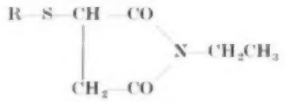
7b


ARG
PRO
LEU
LYS
HIS
VAL
ALA
GLY
TAU
SER
GLU
ASP
CYS

7c

When authentic samples of GSH and of GSSG were submitted to electrophoresis both substances migrated to about the same position as spot P of the tissue extracts. GSSG formed a much more compact spot than did GSH and it had a slightly greater mobility than GSH. However, mixtures of GSH and GSSG invariably gave a single spot. After incorporation of NEM into phosphate buffer solutions of the pure materials, migration of GSSG was unaffected but the spot due to GSH was considerably retarded with respect to GSSG and now occupied position Z′.

Friedmann, Marrian and Simon-Reuss (1949) showed that NEM has a powerful affinity for thiol compounds (RSH) which react by addition at the ethylene bond of NEM to give products of the type :



According to Gregory (1955) the rapid reaction between NEM and GSH can be used for quantitative purposes. Although cysteine and H_2S also react with NEM, Gregory found that no reaction occurred with *S*-acetylglutathione, GSSG, ethanol, ethylamine or HCN. In the present work we have confirmed that reaction of NEM with naturally occurring ninhydrin-positive materials seems to be limited to those substances which contain free thiol groups.

It is concluded that substance X in hepatocarcinogen-treated rat liver and in normal guinea pig-liver is closely related to authentic GSH and that both compounds contain a free thiol group capable of reacting with NEM.

(d) *Attempted isolation of thiol peptide X along with GSH from the livers of rats injected with 3′-MeDAB.*—Attempts were made to isolate thiol peptide X along with GSH from perfused fresh livers of rats injected with 3′-MeDAB by the cadmium chloride-cuprous mercaptide precipitation method of Waelsch and Rittenberg (1941).

Although X was prominent in the 3′-MeDAB livers and virtually absent in the normal control livers (Fig. 1 shows electropherograms of extracts of some of the rat livers used in these experiments) electrophoresis of the final products obtained by the action of H_2S on aqueous suspensions of the cuprous mercaptide prepared from the 3′-MeDAB liver failed to reveal any spot other than that due to authentic GSH. Thus peptide X may be a labile derivative of GSH which readily reverts to the parent substance during the isolation procedure.

Hydrolysis experiments were carried out with an authentic specimen of GSH which had been submitted to the cuprous mercaptide isolation procedure (with 90 per cent recovery) and on GSH specimens prepared as described above from livers of normal and 3′-MeDAB injected rats. No differences could be detected in chromatograms of the hydrolysis products which in each case showed strong spots due to glycine, glutamic acid, cysteine and/or cystine. It is interesting that no cysteic acid was detected chromatographically in experiments in which rather large amounts of the peptides were subjected to hydrolysis. The concentration of GSH in the hydrolysis medium was not without influence on the nature of the hydrolytic products. Thus at a concentration of 4 μg of GSH per 0·1 ml. of 6 N HCl, 3 spots due to glycine, glutamic acid and cysteic acid were detected whereas in hydrolysates containing initially 12 μg of GSH per 0·1 ml. of

6 N HCl appreciable amounts of either cysteine or cystine were detected but only traces of cysteic acid.

A pooled liver sample (15 g. ; 5 g. from each of 3 rats) from rats injected with 3′-MeDAB contained 167 mg. of GSH (regenerated from the cuprous mercaptide) per 100 g. wet weight of liver, whereas a similar sample from 3 normal (arachis oil injected) rats contained 123 mg. of GSH per 100 g. wet weight of liver.

The increase in rat liver GSH following intraperitoneal injection of 3′-MeDAB is in accord with results of Fiala (1958) who showed that rat liver GSH increases in the early stages of feeding this azo dye. A similar increase in the GSH content of mouse liver after intraperitoneal injection of the liver carcinogen, 3, 4 : 5, 6-dibenzcarbazole, was observed by Boyland and Mawson (1938).

(e) *Comparison of the electrophoretic behaviour of various GSH derivatives and related products with that of thiol peptide X.*—Since efforts to isolate substance X had failed, attempts were made to gain further insight into its nature by comparative studies of the electrophoretic behaviour of solutions of known GSH derivatives in pH 7·2 phosphate buffer.

It seemed possible that substance X might be *S*-acetylglutathione since Benesch *et al.* (1960) have stated that this compound gives a colour reaction with NEM which is said to be typical of a number of free thiol compounds and some thiol esters but which is not given by thioethers. A sample of *S*-acetylglutathione had the same mobility as substance X under our electrophoresis conditions. However, as shown in Fig. 5, the mobility of this substance was unaffected by NEM. Evidently, substance X is not *S*-acetylglutathione and it seems probable that the colour reaction given by this substance may in fact be due to GSH formed from the *S*-acetyl derivative in the preliminary reaction with NEM before colour development with KOH. As noted by Gregory (1955) no spectroscopic evidence could be obtained for a reaction between *S*-acetylglutathione and NEM.

It was of interest to compare the electrophoretic behaviour of *S*-methylglutathione with that of *S*-acetylglutathione although the former was unlikely to be identical with tissue substance X. *S*-methylglutathione was prepared by the method of Kermack and Matheson (1957). Although this compound had a lower mobility than GSH and about the same mobility as substance X it failed to react with NEM. Similarly a specimen of *S*-benzylglutathione prepared as described by Saunders (1934) had about the same mobility as X and was uninfluenced by NEM. Thus when the thiol group of GSH is blocked by the groups acetyl, methyl or benzyl the mobility of these derivatives is retarded as compared with authentic GSH to about the same extent as X. Unlike X however, they failed to react with NEM. It seems certain that the thiol group of peptide X is free.

Although hydrolytic studies have shown that peptide X apparently contains the three amino acids, glycine, glutamic acid and cysteine, it seemed worthwhile to check the electrophoretic behaviour of the dipeptides γ-glutamyl-cysteine and cysteinylglycine. These substances were prepared by methods given by Gutcho and Laufer (1954). γ-glutamylcysteine had about the same mobility as glutamic acid. It reacted with NEM to give a single strong ninhydrin-positive spot of diminished mobility at about position Z′. Similar results were obtained with cysteinylglycine. Neither of these thiol dipeptides had the mobility of thiol peptide X.

An indication that substance X might be an abnormal oxidation product of GSH is contained in a paper by Blass, Le Comte and Polonovski (1954). These

authors noted 3 spots in electropherograms of GSH which had been treated with hydrogen peroxide under unspecified conditions. One of the oxidation products (probably GSO_3H) lay ahead of the main GSH spot (probably GSSG) in the anode direction while the other had a lower mobility than the main spot. Judging from a diagram of the separation presented in their paper it appeared that the oxidation product of low mobility had about the same mobility as substance X. Although we were unable to detect any of the slow moving substance in a specimen of GSH treated with hydrogen peroxide, we did observe that an old specimen of GSH (not treated with H_2O_2) gave a strong ninhydrin-positive spot in the usual position for GSH and two slowly-developing weaker spots: a compact spot with greater mobility than GSH and a diffuse spot with a lower mobility than the main spot. However the mobility of the slow spot was rather less than that of tissue substance X and was about equal to that of the GSH–NEM reaction product Z'. The fast spot disappeared when NEM was added to the GSH stock solution but the mobility of the slow spot was unaffected. Thus the fast material probably contains a free thiol group whereas the slow moving spot evidently contains no such group.

It seemed of interest to investigate oxidation products of GSH arising in other experimental situations and to this end electrophoretic studies were made of ninhydrin-positive products resulting from (i) irradiation of a solution of GSH in phosphate buffer (pH 7·2) with ultraviolet light and from (ii) a reaction between GSH and napthalene-2-sulphonyl chloride (N–2–SO_2Cl).

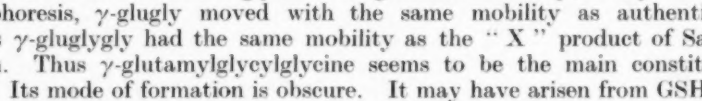
(i) Woodward (1933) found that GSH was oxidised mainly to GSSG by ultraviolet light at pH 7 but some degradation of GSH also occurred. This reaction was reinvestigated with a solution of GSH (10 mg.) in pH 7·2 phosphate buffer (4 ml.) which was exposed in a 1 cm. quartz cell for 4 hours at 20° C. to unshielded light from the mercury arc of an Hanovia lamp, the mercury arc being about 6 inches from the cell. A suitable dilution of the irradiated solution (which had a strong smell of H_2S) was prepared with pH 7·2 buffer and aliquots with and without NEM were submitted to paper electrophoresis. Fig. 6A shows a typical separation of ninhydrin-positive materials resulting from this reaction (GSH + UV). For reference, GSH and its NEM reaction product have been included in the electropherogram. The main spot marked " GSH " seems to consist entirely of GSSG since its intensity was unchanged in a parallel run including NEM (not shown in Fig. 6) and no new spot of lower mobility appeared. A second prominent spot " X " having a lower mobility than the " GSH " spot occurred in the region generally occupied by tissue X spot. Its intensity and position were unaltered by NEM. Chromatographic analyses were made of unhydrolysed and hydrolysed " GSH " and " X " materials. Spot " GSH " contained only glutamic acid, glycine and cysteic acid and the chromatogram of the hydrolysate was identical with that shown in Fig. 2C for authentic GSH. A control analysis of authentic GSSG gave the same results. However it is clear from Fig. 6B and 6C that the substance " X " does not correspond to tissue substance X. It gave a single strong purple spot with an R_f value of 0·37 in phenol (Fig. 6B). On hydrolysis, 3 spots were obtained corresponding to glutamic acid, glycine and α-alanine with Rf values in phenol of 0·28, 0·39 and 0·60 respectively (Fig. 6C). Thus " X " could be identical with the tripeptide γ-glutamylalanylglycine which has been detected by Waley (1957) in bovine lens. DL–γ–glutamyl–DL–alanylglycine was synthesised according to Kermack and Matheson (1957) and subjected to paper electrophoresis, elution and chromatographic analysis as

already described. It had the same electrophoretic mobility as peptide " X ". The unhydrolysed material had Rf 0·39 in phenol, and, on hydrolysis the peptide gave 3 spots due to glutamic acid, glycine and α-alanine with Rf values of 0·30, 0·42 and 0·62 respectively in phenol and the spots had the same relative intensities as the spots seen in Fig. 6C. It is concluded that peptide " X " is γ-glutamyl-alanylglycine. It probably arose by loss of H_2S from the cysteinyl residue of GSH with subsequent reduction of the resulting dehydroalanyl tripeptide to the alanyl derivative.

(ii) Saunders (1933) investigated a peculiar reaction which occurred between GSH and naphthalene-2-sulphonylchloride (N–SO_2Cl or NSC). The latter failed to react in the ordinary way with the amino group of GSH. Instead it promoted an oxidation reaction and was itself reduced to naphthalene-2-sulphinic acid (N–2–SO_2H or NSH). On stoichiometric grounds Saunders concluded that the main reaction product ought to be GSO_2SG but his analysis of the ill-defined product failed to support this view.

This reaction was reinvestigated and after removal of NSH by precipitation with HCl, deep freezing and centrifugation, a portion of the yellowish acidic supernatant smelling strongly of H_2S was diluted with phosphate buffer (pH 7·2) and examined electrophoretically. A typical separation is shown in Fig. 6A (GSH + NSC). Analysis of the main spot " GSH " revealed that it probably consists mainly of GSSG. Addition of NEM to the phosphate buffer solution resulted in the development of a Z′ spot, showing that some free thiol compound, probably GSH, is still present in the reaction mixture. As in the case of GSH + UV there appeared a very faint red spot just on the cathode side of the origin in the position to which authentic glycine migrates. These glycine spots cannot be seen clearly in the autopositive reproduction, Fig. 6A.

The nature of the " X " spot observed in this separation is of considerable interest although it is evidently quite different from tissue X spot. The unhydrolysed substance (Fig. 6D) gave a purple ninhydrin-positive spot with Rf0·32 in phenol. After acid hydrolysis, 2 spots were found in the chromatograms with Rf values in phenol of 0·28 and 0·39 due to glutamic acid and glycine respectively. Thus " X " is evidently a peptide consisting of glutamic acid and glycine. It could be either γ-glutamylglycine (γ-glugly) or γ-glutamylglycylglycine (γ-gluglygly). It is unlikely to be glycylglutamic acid since this substance would be expected to give a yellow colour with ninhydrin as do the glycyl dipeptides of glycine, serine and valine (Bowden, 1959). Samples of γ-glugly and γ-gluglygly were prepared by methods of King and Kidd (1949) and Kermack and Matheson (1957). These substances gave strong purple ninhydrin-positive spots with Rf values of 0·32 (glugly) and 0·29 (gluglygly) in phenol. After acid hydrolysis, γ-gluglygly gave two spots with Rf values in phenol of 0·39 and 0·27 due to glycine and glutamlc acid respectively. On paper electrophoresis, γ-glugly moved with the same mobility as authentic GSH whereas γ-gluglygly had the same mobility as the " X " product of Saunder's reaction. Thus γ-glutamylglycylglycine seems to be the main constituent of " X ". Its mode of formation is obscure. It may have arisen from GSH by the following reaction sequence :



It seems less probable that γ-gluglygly could have been formed in a transpeptidation reaction. In biochemical systems, γ-glutamylcysteine can transfer its glutamyl group to other amino acids present in the medium. For instance, Talalay (1954) observed the formation of γ-glutamylglycine and γ-glutamylglutamic acid in a solution of GSH (pH 7·4) which had been incubated for 1 hr at 37° C. with *Proteus vulgaris* or with cell-free extracts of this organism. Since traces of glycine have been detected among the products of reaction of GSH with NSC, γ-glutamylcysteine may be presumed to have been present at some stage in the reaction. Further work will be required to determine the precise mode of formation of γ-gluglygly in the NSC *in vitro* system.

Thus we have obtained no definite evidence for the occurrence *in vitro* of an oxidation product of GSH resembling tissue substance X. It will be noted however that traces of materials behaving like tissue substance X or GSH can be seen in Fig. 6, B, C, D and E. These traces may have arisen in the *in vitro* reactions described above but it seems more likely that they are due to small amounts of GSH which have been carried over from the main "GSH" spot in the paper sections containing "X" spot as selected for chromatographic analysis.

(f) *Stability of thiol peptide X in stored deep frozen phosphate extracts and tissues.*—Although a ninhydrin positive X spot can still be detected in electropherograms of aged extracts of hepatocarcinogen-treated rat livers and tumours, the mobility of the substance is no longer affected by NEM. An example of this can be seen in Fig. 7A. Apparently the thiol group has become masked probably through oxidation to disulphide or mixed disulphide. In view of the similar mobilities of GSH and GSSG under our conditions it was reasonable to conclude that XSH and XSSX would also have about the same mobilities.

An acid hydrolysate of X from a 2-month old extract of tannic acid-treated rat liver showed definite traces of aspartic acid (turquoise colour with ninhydrin), alanine and glycine as well as prominent spots due to glutamic and cysteic acids. A similar picture was given by an hydrolysate of X obtained from a benzopyrene-induced, tumour, with the exception that a spot corresponding to serine rather than to alanine appeared in the chromatograms.

Wieland (1954) has noted that alanine may arise in hydrolysates of GSSG (in the present work, however, alanine was never observed in hydrolysates of authentic GSSG) as well as the expected spots due to glycine, glutamic acid and cysteine. The occurrence of aspartic acid and serine in some of the aged X products suggests the formation of mixed disulphides of XSH with thiol peptides containing aspartic acid or serine. No evidence has been obtained for the presence of such peptides in any of our extracts nor have we observed the formation of any ninhydrin-positive products with themobility of X in stored deep frozen extracts of normal livers which were free of X when fresh.

Most of the stored extracts had been thawed, exposed briefly to the air and refrozen several times, factors which may have contributed to changes in X. The question as to whether X is a metabolically active material related to GSH seems to deserve further study, but it must not be forgotten that the presence of aspartic acid etc. might be explained on the basis of the *in vitro* formation of ninhydrin-negative substances such as acetyl-aspartic acid having the same mobility as X.

X, or a substance of similar mobility, has been found to survive for at least

several months in deep frozen livers of hepatocarcinogen-treated rats. On the other hand, a specimen of normal guinea-pig liver which initially gave a strong X spot lost this material after several weeks at −15° C. It is of interest that substance X from a normal guinea-pig liver extract which had been stored for 10 days at −15° C. still responded completely to the addition of NEM. However, the intensity of the PE + GSH spot was unaffected by the NEM treatment. Evidently all the GSH had been converted to GSSG whereas the thiol group of substance X remained free.

2. *Nature of Y spot*

This small spot with mobility greater than that of aspartic acid was sometimes found in hepatocarcinogen-treated rat liver extracts and invariably in rat tumour extracts. It has not been extensively studied. It disappeared from electropherograms of an Rd/3 tumour phosphate extract to which NEM had been added, and the same effect was found with Y spot from normal guinea-pig liver extract. It has already been noted that a small spot with mobility was detected in electropherograms of an old specimen of GSH and similar products were observed in GSH solutions subjected to ultraviolet irradiation or to the action of naphthalene-2-sulphonyl chloride. These spots had about the same mobility as Y and disappeared when NEM was added.

A brownish spot appeared in the region of Y after spraying the electropherogram from tannic acid-treated rat liver phosphate buffer extract with aniline hydrogen phthalate spray (Partridge, 1949). This result suggests that a sugar-containing residue may be present in close association with Y.

3. *Nature of Z spot in various rat tumours, in the livers of tumour-bearing rats and in normal guinea-pig liver*

Extracts of all rat tumours investigated have been found to contain a ninhydrin-positive spot Z, examples of which are shown in Fig. 4 and in Fig. 7A.

Products of hydrolysis of Z spot from the BP tumour extract shown in Fig. 7A were chromatographed. The hydrolysate (Fig. 7B) contained appreciable amounts of aspartic, glutamic and cysteic acids and lesser amounts of ninhydrin-positive spots which have been tentatively identified as serine, glycine, proline, α-alanine, valine, leucine, lysine and possibly arginine and histidine (compare reference chromatogram Fig. 7C). Practically the same amino acid picture was given by substance Z isolated from electropherograms of extracts of a rapidly growing Rd/3 tumour or from a slow-growing DMBA tumour.

Unhydrolysed extracts of Z from the three types of rat tumour were submitted to chromatography. In each case no spots due to free amino acids were detected All that could be seen was a very minute intensely purple spot running at the phenol front and having zero Rf in the butanol direction. Thus Z is evidently a protein or polypeptide-like material composed of at least 10 to 12 amino acids.

As already mentioned spots corresponding to Z were found in the livers of rats bearing transplantable tumours, in normal guinea-pig liver and also in normal rat kidney. However, when these spots were eluted and chromatographed there always appeared a single strong reddish ninhydrin-positive spot with Rf values similar to those of cysteine, as well as a minute purple spot running at the phenol front. When liver or kidney Z spots were hydrolysed and chromatographed

an amino acid picture qualitatively rather similar to that given by tumour Z spot was obtained. The new spots apparently arose from the polypeptide moving at the phenol front. It was noted that the cysteic acid spot in hydrolysates of these Z spots was much more intense than in hydrolysates of material taken from a region lying on the origin side of Z spot. Unhydrolysed material from this position contained a polypeptide spot but no cysteine.

In spite of the rather sharp separation indicated in Fig. 7A it has been difficult to determine to what extent tumour Z spot has been contaminated with ninhydrin-positive material tailing from the origin towards Z spot. However it has been found possible to remove Z spot as well as all other amino acids spots by washing the electropherograms with several changes of deionised water. There remains a tail of material directed towards the anode from the origin which is revealed by staining with mercuric chloride-bromophenol blue or with ninhydrin. It probably consists of protein.

The compact and isolated nature of Z spot as seen in Fig. 7A suggests that Z spot is in fact a definite polypeptide entity unassociated with free amino acids. It is certain that tumour Z spot cannot be due merely to the presence of a free amino acid such as cysteine on a background of polypeptide or protein as seems to be the case with the Z spot material from other tissues.

DISCUSSION

A thiol peptide X has been detected in the livers of rats injected with hepatocarcinogens at times when the free phosphoethanolamine content of the liver is maximal. Normal guinea-pig liver in contrast to normal rat liver already contains appreciable amounts of a substance similar to and apparently identical with X. Thiol peptide X contains the same amino acids as GSH but differs from GSH in having a lower electrophoretic mobility. X might be an isomer of GSH, a homologue of GSH, a higher peptide with the same amino acid constituents as GSH, or a derivative of GSH with some labile group. Various possibilities, some of which are being studied in detail, are outlined below.

With regard to possible isomers of GSH in which the mode of linkage of the constituent amino acids has been altered, little seems to be known. Du Vigneaud, Loring and Miller (1937) synthesised α-glutamylcysteinyl-glycine. However this product (not known to occur naturally) retained its identity after liberation from its insoluble cuprous mercaptide complex. In taking account of optical isomers of GSH which might possibly have electrophoretic properties different from that of naturally occurring GSH, L-γ-glutamyl-L-cysteinylglycine, it is noted that the isomer D-γ-glutamyl-L-cysteinylglycine has been synthesised by Kögl and Akkerman (1946). The authors could find no polarimetric evidence for the occurrence of this substance in liver or tumour tissue. The fact that the D-isomer could be isolated unchanged from its insoluble cuprous mercaptide makes it unlikely that tissue substance X could be identical with this substance.

Ball, Revel and Cooper (1956) considered that γ-glutamylglutathione might be an intermediate in some transpeptidation reactions of GSH but apparently this peptide has not been isolated from biochemical systems nor has it been synthesised. It might fulfil the requirements for tissue substance X and indeed, after making allowance for the barbitone hydrolysis artifact, it would appear

that the glutamic acid spot is more intense than either the glycine or cysteic acid spot in hydrolysates of X as compared with hydrolysates of authentic GSH (Fig. 2). In preliminary studies we have been able to effect separation of peptide X from GSH in the volatile component (pyridine-acetic acid-water) electrolyte recommended by Jirgl (1959). It is hoped with this method to obtain more exact information about the proportions of the constituent amino acids of X without interference from buffer artifacts.

The preponderance of glutamic acid in X might be due to the presence in the spot of a ninhydrin-negative glutamic acid derivative having the same mobility as X. One such contaminant might be pteroylglutamic (folic) acid which, on electrophoresis, separates into two fluorescent components which travel towards the anode. One of these components had the same mobility as X. It is quite possible that such a contaminant of X would only reveal itself by its contribution of extra glutamic acid in chromatograms of X hydrolysate.

Although a homologue of GSH (GCH_2SH) containing homocysteine in place of cysteine has been synthesised (Herrick and Todd, 1955; Gawron and Draus, 1959) this substance has apparently not been found in biochemical systems. Such a compound with an extra $-CH_2-$ group in the thiol side chain might well have properties similar to X. It might not be possible to distinguish GCH_2SH from GSH by chromatography of its hydrolysis products since homocysteic acid has about the same Rf values as cysteic acid in Bowden's system. Like GSH, the homologue would be expected to form an insoluble cuprous mercaptide. However, Herrick and Todd (1955) found that the cuprous mercaptide of L-γ-glutamyl-DL-homocysteinylglycine is in fact easily soluble in water so the possibility cannot be ruled out that thiol peptide X, if not GCH_2SH, may at least be capable of forming a water-soluble cuprous derivative and so be lost in the cuprous mercaptide precipitation procedure.

There has been speculation as to the existence of a variety of interrelated GSH derivatives including compounds with pyrrolidine and thiazoline rings (Calvin, 1954; Isherwood, 1959). Pyrrolidine-imonium (PI) or hydroxypyrrolidine (HP) forms of GSH proposed by Calvin (1954) have free thiol groups but the α-amino group of the glutamyl residue has reacted with the γ-carbonyl group with loss of water (PI) or by simple addition (HP). According to Calvin, HP could readily revert to the normal open chain form of GSH in the presence of hydrogen ions. Thus the reaction of tissue substance X with NEM, its colour reaction with ninhydrin-acetic acid and the apparent disappearance of X in the acidic conditions required for cuprous mercaptide formation could be accounted for if X possessed the hydroxypyrrolidine structure.

Thiol peptide X might be a GSH derivative with a substituent not readily detectable chromatographically or easily lost during handling. Dubin and Rosenthal (1960) reported the isolation of a conjugate of GSH with spermidine from *E. coli*. From the available information it would appear that this complex consists of a molecule of GSSG which has reacted through its COOH groups with the amino groups of spermidine. The conjugate ran as a single spot but after hydrolysis it gave four spots due to glycine, glutamic acid, cysteic acid and spermidine. In view of the elevated levels of PE which occur in hepatocarcinogen-treated rat liver it seemed that X might be a similar conjugate of GSH with PE or ethanolamine. No chromatographic evidence has yet been obtained in favour of the existence of such a conjugate, but it is highly likely that PE or ethanolamine

residues would fail to survive the hydrolytic and freeze-drying procedures employed in the present study.

There are some indications in the literature that substances like thiol peptide X have been detected in other situations. Thus Spicer and Weise (1955) reported that a cationic fraction of guinea-pig or rabbit liver, submitted to paper electrophoresis at pH 4·6, gave two unidentified ninhydrin-positive spots which stood in the same relation to GSH, glutamic and aspartic acids as did substances X and Y of the present study.

Waley (1956) found that an acidic fraction of calf lens contained several peptides consisting of glutamic acid, cystine (or cysteine) and glycine. These materials were not identical with GSH and they appeared to contain differing molar ratios of glycine to glutamic acid. Waley considered that one of these peptides might carry a phosphate group.

Keir and Davidson (1956) reported that the cytidine monophosphate fraction of rabbit tissues invariably gave a reaction with ninhydrin. When the fraction was submitted to electrophoresis the ninhydrin-positive constituent moved ahead of cytidine monophosphate. On hydrolysis, the unknown constituent yielded glutamic acid, cystine and glycine together with an unidentified base which absorbed ultraviolet light.

Whatever the nature of X and Y may be, it appears that X at least may result from some disruption of the normal course of GSH metabolism in rat liver through the influence of hepatocarcinogens or by the presence of a remote tumour. Substances X and Y may themselves play some part in tumour metabolism. X may be a normal trace derivative of GSH which appears in increased amount in rat liver under the stress conditions imposed by hepatocarcinogens or tumours. Schacter and Law (1956) found that there is an increased requirement for GSH from the liver, accompanied by an increased output of GSH by the liver, during the growth of an ascitic form of lymphocytic leukaemia in mice.

It is of considerable interest that a substance similar to thiol peptide X is already present in appreciable amounts in the liver of normal guinea-pig, a species which is refractory to hepatocarcinogenesis. Moreover, chromatographic evidence has been obtained that 3′MeDAB injection failed to increase the level of free PE in guinea-pig liver (Neish, unpublished results) whereas this hepatocarcinogen induced a marked increase of PE in rat liver. These pronounced differences in behaviour of X and PE in the livers of rat and guinea-pig may well be connected with the susceptibility of the former and the refractoriness of the latter species to hepatocarcinogenesis.

A significant relationship seems possible between the high level of GSH in hepatocarcinogen-treated rat liver (at least in the initial stages ; Fiala, 1958) and the elevated PE content. Henneman, Altschule and Goucz (1955) found a decrease in the level of inorganic phosphate in the serum after injection of GSH into humans which suggests that the high level of GSH may be promoting phosphorylation reactions. Conversely, according to Jocelyn (1959) low GSH levels may depress phosphorylation. Some evidence for this is found in the work of Ling and Chow (1954) who showed that the blood and tissue levels of phospholipids are abnormally low in Vitamin B_{12}-deficient (low GSH) rats. It is interesting that the enzyme alkaline phosphatase which catalyses the hydrolysis of PE (PE may be the natural substrate for this enzyme, according to Dent, 1956) is said to activated by GSSG and inhibited by GSH (Fell and Danielli, 1944).

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Thus the increased level of GSH in the liver of rats injected with 3′-MeDAB might be expected to favour the accumulation of PE which has in fact been observed (Neish and Rylett, 1960).

Vollmer (1954) stated that GSH is lost from red blood cells in the presence of methaemoglobinogenic agents. Since it is known that 3′-MeDAB induces methaemoglobinemia in the rat (Neish, 1956) it seemed possible that the formation of thiol peptide X in rat liver might be connected with some disturbance in liver GSH metabolism associated with the methaemoglobinogenic action of the azo dye. However, such an effect can probably be ruled out because the equally potent hepatocarcinogen, 4′-EtDAB, behaves exactly like 3′-MeDAB with regard to the accumulation of PE and the formation of thiol peptide X in rat liver although, compared with 3′-MeDAB on a mole for mole basis, it has no methaemoglobinogenic activity in the rat (Neish, 1959).

The substance Z which has been found in extracts of rapid and slow growing transplantable rat tumours and of benzopyrene-induced rat tumours seems to be a protein or polypeptide. Borsook *et al.* (1949) obtained a polypeptide consisting of 15 amino acids from the livers of various species. Several acidic peptide-nucleotide complexes have been encountered by Steinberg *et al.* (1960) in mammalian liver and Ågren (1960) has obtained an acid-soluble nucleotide-linked peptide from trichloracetic acid extracts of ascites tumour cells. On hydrolysis, this peptide gave particularly strong gly, glu, ser and asp spots, weaker spots due to leu, iso-leu, lys, arg, threo and at least 4 more spots which were not identified.

Tumour substance Z may be compared with the toxohormone of Nakahara and Fukuoka (1958). Toxohormone is said to be produced by all malignant tumours and its major amino acid components are stated to be ala, pro, asp, arg, Øala, lys, leu and glu. A toxohormone-like material isolated from the urine of cancer patients by Fuchigami, Umeda and Ono (1956) contained asp, glu, ser, threo, pro, ala, lys, val, leu, Øala, cystine and gly all of which with the exception of threo and Øala appear to occur in Z. Whether Z further resembles toxohormone in having catalase-inhibiting properties remains to be determined.

It is of interest that hepatocarcinogens induce the formation of a thiol peptide X and of excessive amounts of phosphoethanolamine in rat liver. Rat sarcomas appear to have a higher level of PE than normal rat muscle tissue (Neish, unpublished results) and they also contain thiol peptide X which is not present in normal muscle. These observations suggest that it might be worthwhile to examine the possibility of the emergence of tumour-type metabolic patterns even in the early stages of the action of a carcinogen on its target organ. Recently, Kögl *et al.* (1960) stated that the lecithin fraction of primary rat tumours induced by 4-dimethylaminoazobenzene incorporated considerably more ^{32}P *in vitro* than did the phosphatidylethanolamine fraction. The opposite situation was found with normal liver tissue. Thus a shift in the metabolic activities of both phosphatides appears to occur during development of liver tumours. It has already been noted (Neish and Rylett, 1960) that a possible explanation for the accumulation of PE in the livers of hepatocarcinogen-injected rats lies in a carcinogen-induced failure of the enzymic mechanism for phosphatidylethanolamine synthesis. Kögl's work suggests that a similar defect may be present in the azo-dye induced tumours. Thus an early effect of the carcinogen may persist through the course of hepatocarcinogenesis.

SUMMARY

1. The livers of hepatocarcinogen-treated rats and of normal guinea-pigs contain a substance X which is apparently a thiol peptide closely related to glutathione. A similar substance has been detected in transplantable and induced rat tumours together with a substance Y which may also be a thiol peptide. Attempts to characterise X fully have not yet been successful but some pointers towards its possible nature have been obtained.

2. All the rat tumours investigated contained a substance Z which seems to be a protein or polypeptide containing at least 10 to 12 amino acids.

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REFERENCES

Ågren, G.—(1960) *Acta chem. scand.*, **14**, 2065.
Ball, E. G., Revel, J. P. and Cooper, O.—(1956) *J. biol. Chem.*, **221**, 895.
Benesch, R., Benesch, R. E., Gutcho, M. and Laufer, L.—(1960) *Science*, **123**, 981.
Blass, J., Le Comte, O. and Polonovski, J.—(1954) *Bull. Soc. Chim. biol., Paris*, **36**, 627.
Borsook, H., Deasy, C. L., Haagen-smit, A. J., Keighley, G. and Lowy, P. H.—(1949) *J. biol. Chem.*, **179**, 705.
Bowden, C. H.—(1959) *Clin. Chim. Acta*, **4**, 539.
Bowland, E. and Mawson, E. H.—(1938) *Biochem. J.*, **32**, 1460.
Calvin, M.—(1954) 'Glutathione'. Ed. S. Colowick *et al.* New York (Academic Press Inc.), p. 3.
Dent, C. E.—(1956) Ciba Symposium—Bone Structure and Metabolism. London (Churchill), p. 266.
Dubin, D. T. and Rosenthal, S. M.—(1960) *Fed. Proc.*, **19**, Part 1, p. 1.
Du Vigneaud, V., Loring, H. S. and Miller, G. L.—(1937) *J. biol. Chem.*, **118**, 391.
Fell, H. B. and Danielli, J. F.—(1944) *Bull. War Med.*, **4**, 253.
Fiala, S.—(1958) *Nature, Lond.*, **182**, 257.
Friedmann, E., Marrian, D. H. and Simon-Reuss, I.—(1949) *Brit. J. Pharmacol.*, **4**, 105.
Fuchigami, A., Umeda, M. and Ono, T.—(1956) *Gann.*, **47**, 295.
Gavron, O. and Draus, F.—(1959) *J. org. Chem.*, **24**, 1392.
Gregory, J. D.—(1955) *J. Amer. chem. Soc.*, **77**, 3922.
Gutcho, M. and Laufer, L.—(1954) 'Glutathione'. Ed. S. Colowick *et al.* New York (Academic Press Inc.), p. 79.
Heineman, D. H., Altschule, M. D. and Goucz, R. M.—(1955) *Metabolism*, **4**, 433.
Herrick, E. C. and Todd, C. W.—(1955) *U.S. Pat. Syst. Leafl.*, No. 2,723,972 and 2,723,973.
Isherwood, F. A.—(1959) *Biochem. Soc. Symp.* No. 17, Glutathione. London (Cambridge University Press), p. 13.
Jirgl, V.—(1959) *Experientia*, **15**, 235.
Jocelyn, P. C.—(1959) *Biochem. Soc. Symp.* No. 17, Glutathione. London (Cambridge University Press), p. 60.
Keir, H. M. and Davidson, J. N.—(1956) *Biochem. J.*, **63**, 23P.
Kermack, W. O. and Matheson, N. A.—(1957) *Ibid.*, **65**, 45.
King, F. E. and Kidd, D. A. A.—(1949) *J. chem. Soc.*, p. 3315.

UMI

KÖGL, F. AND AKKERMAN, A. M.—(1946) *Rec. Trav. chim. Pays-bas*, **65**, 216, 225.
Idem, SMAK, C., VEERKAMP, J. H. AND VAN DEENEN, L. L. M.—(1960) *Z. Krebsforsch.*, **63**, 558.
LING, C. T. AND CHOW, B. F.—(1954) *J. biol. Chem.*, **206**, 797.
NAKAHARA, W. AND FUKUOKA, F.—(1958) *Advanc. Cancer Res.*, **5**, 157.
NEISH, W. J. P.—(1956) *Nature, Lond.*, **178**, 1350.—(1959) *Naturwissenschaften*, **46**, 535.
Idem AND RYLETT, A.—(1960) *Brit. J. Cancer*, **14**, 737.
PARTRIDGE, S. M.—(1949) *Nature, Lond.*, **164**, 443.
SAUBERLICH, H. E. AND BAUMANN, C. A.—(1951) *Cancer Res.*, **11**, 67.
SAUNDERS, B. C.—(1933) *Biochem. J.*, **27**, 397.—(1934) *Ibid.*, **28**, 1977.
SCHACTER, B. AND LAW, L. W.—(1956) *J. nat. Cancer Inst.*, **17**, 391.
SPICER, S. S. AND WEISE, V.—(1955) *Enzymologia*, **17**, 263.
STEINBERG, D., VAUGHAN, M., SHERMAN, F. G. AND O'DELL, B. L.—(1960) *Biochim. biophys. Acta*, **40**, 225.
TALALAY, P. S.—(1954) *Nature, Lond.*, **174**, 516.
VOLLMER, E. P.—(1954) 'Glutathione'. Ed. S. Colowick *et al.* New York (Academic Press Inc.), p. 186.
WAELSCH, H. AND RITTENBERG, D.—(1941) *J. biol. Chem.*, **139**, 761.
WALEY, S. G.—(1956) *Biochem. J.*, **64**, 715.—(1957) *Ibid.*, **67**, 172.
WIELAND, T.—(1954) 'Glutathione'. Ed. S. Colowick *et al.* New York (Academic Press Inc.), p. 55.
WOODWARD, G. E.—(1933) *Biochem. J.*, **27**, 1411.

DISTRIBUTION OF ^{65}Zn IN THE PROSTATE AND OTHER ORGANS OF MAN

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ALTHOUGH there are many biological studies dealing with zinc, little quantiative data have appeared regarding the concentration and turnover of this metal, especially for man. Interest in zinc metabolism has been stimulated by the accumulating evidence which indicates that this metal is a constituent of several enzymes (Fischer, Tikkala and Mawson, 1955; Vallee, 1955) and is associated, also, with some pathological states (Daniel *et al.*, 1956; Prout, Sierp and Whitmore, 1959; Herring *et al.*, 1961; Fredricks, Tanaka and Valentine, 1960). Our concern with the element stems from reports of the high concentration of zinc found in the prostate gland both in animals (Gunn *et al.*, 1955) and man (Mawson and Fischer, 1952).

While its biological significance is established, the physiological role of zinc remains to be elucidated. Important contributions to our understanding of the part played by the element should result from investigations which employ ^{65}Zn. Precise data are not yet available as to the fraction of the administered radioisotope which is taken up by the various organs. Quantitative concentration data are unavailable for the prostate; moreover, the particular sites and structures of the gland associated with ^{65}Zn uptake have not been identified.

Following our earlier study of the distribution of ^{65}Zn in blood and its excretion in man (Graig and Siegel, 1960), the present investigation was undertaken to learn more about the concentration of the radioisotope by several organs with special attention to the prostate. For the latter, it was also sought to ascertain which glandular components are implicated in the accumulation of ^{65}Zn.

MATERIALS AND METHODS

Autopsy studies.—Fourteen pre-terminal patients having various malignancies were given approximately 100 μc ^{65}Zn (specific activity greater than 75 mc/g.) as the chloride. The radioisotope was administered as an intravenous infusion in a 250 ml. volume of isotonic solution. At autopsy, which occurred between 1 and 174 days after the infusion, portions of the following organs were obtained, whenever possible, for digestion and the assay of ^{65}Zn: liver, pancreas, spleen, prostate, seminal vesicles, lung, bladder and skeletal muscle.

Studies on prostatic surgical specimens.—Tracer doses of ^{65}Zn (approximately 50 μc) were administered in the manner described above to 36 patients presumed to have diseased prostates and awaiting surgery. A sample of each surgical

specimen was assayed subsequently for ^{65}Zn concentration, using the technique outlined below. The variaton of radiozinc localization within the prostate itself was investigated in 15 of these patients having benign hypertrophy. Multiple samples were taken from the different lobes (left, right, medial, anterior and posterior) of the gland for determination of ^{65}Zn.

Digestion of organs.—Duplicate weighed samples of each specimen were covered and digested with 20 per cent NaOH in a chemical hood employing moderate heat for several hours. Throughout this period, the level of liquid was maintained by the addition of distilled water. To determine ^{65}Zn concentration (per cent dose/g.), aliquots were taken from the resulting suspension for assay.

Recovery experiments were performed on freshly killed mouse organs to check the reliability of this digestion technique. Known activities of ^{65}Zn (6·3 μc contained in 1 ml.) were added to duplicate samples of each of the following animal organs: brain, lung, spleen, heart, liver, pancreas, kidney, adrenal, muscle and skin. As indicated in Table I, the results justified employing this method for the processing of the various human samples.

TABLE I.—*Results of ^{65}Zn Recovery Experiments, Following NaOH Digestion of Various Mouse Organs with Added ^{65}Zn*

Organ	Per cent ^{65}Zn recovered
Adrenal	99·5
Brain	104·3
Heart	100·5
Kidney	101·5
Liver	99·5
Lung	100·0
Muscle	100·0
Pancreas	99·5
Spleen	100·5
Skin	99·5
	100·5 ± 1·6*

* Standard deviation.

Assay of ^{65}Zn.—Counting of organ suspensions was performed with a well-type scintillation counter, having a 1½ inch diameter NaI-thallium activated crystal. This detector has a sensitivity of $2{\cdot}92 \times 10^5$ cpm/μc for ^{65}Zn, established by calibration against National Bureau of Standards reference solutions. Corrections for physical decay of ^{65}Zn (half-life 244·4 days) were made either from long term decay curves or from concurrent measurements of aliquots of dose solutions. Adequate counts were collected to assure an accuracy of better than 5 per cent.

IN VIVO *counting of prostate.*—The localization of ^{65}Zn by the intact prostate was attempted by using shielded rectal detectors possessing high gamma sensitivity. An Antol type 302 Geiger counter was inserted into a 4 mm. thick lead housing, whose external diameter was 17·5 mm. With a 1 cm. port at the distal end, this counting arrangement had a sensitivity of 100 cpm/μc of ^{65}Zn in contact with a source whose volume was 1 ml. More recently, a Nuclear-Chicago DS-8 scintillation probe was used similarly; its sensitivity with a 6 mm. diameter needle probe was 6700 cpm/μc in contact.

Radioautography

(a) *Prostatic sections.*—To study the localization of the administered radioisotope, radioautographs were prepared from 5–10 micron sections of prostate specimens obtained either at surgery or at autopsy. The organ samples were fixed overnight in absolute methyl alcohol to minimize loss of ^{65}Zn and then cleared in xylene. Unstained sections were coated with Kodak Ltd. AR-10 stripping film (Pelc, 1956), exposed for periods ranging between 14 and 250 days at 4°C. in the presence of a desiccant, after which they were developed for 8 minutes in D-19 developer at 18°C. Following fixing and washing, the radioautographs were stained either with hematoxylin and eosin or with methyl green-pyronin. To check for artifacts, control radioautographs were prepared from non-radioactive prostatic sections.

(b) *Bone marrow smears.*—About a week after the infusion of ^{65}Zn tracer doses, bone marrow aspirates from ten patients having no known hematological disorders were smeared in the darkroom on Kodak NTB plates and fixed with absolute methyl alcohol (Boyd, 1955). Following exposure for over 200 days and photographic processing, the plates were stained with Giemsa.

RESULTS

Distribution of ^{65}Zn in various organs and tissues

Widely different concentrations of radiozinc were found in the organs obtained at autopsy (Table II). The highest levels are those of the liver, reaching about 0·050 per cent dose/g. (wet weight) within a few days following administration of the tagged metal. These values for the concentration in the liver are about 2–3 times greater than those in the pancreas, the next most active organ, and as much as 10–30 times the levels in muscle, the tissue with the lowest concentration in our study. Turnover of ^{65}Zn in the liver is apparently slow; as late as 81 days following infusion of the radioisotope, the concentration is still about 0·013 per cent dose/g. The radioisotope is handled more rapidly by the pancreas, so that the maximum concentration, about 0·023 per cent dose/g., is attained by the second day. Within a week, the ^{65}Zn level of the pancreas is reduced by two-thirds, and by 81 days, hardly a tenth remains. The maximum ^{65}Zn concentration in the spleen, about half that of the pancreas, occurred on the third day. The small amounts of radiozinc in the seminal vesicles, bladder and muscle do not appear to change significantly with time.

The radioautographs prepared from the bone marrow smears (Fig. 1–6) indicate that the greatest amounts of ^{65}Zn are taken up by all early forms of the hematopoietic series. Megakaryocytes are also very heavily labeled. Mature leucocytes take up lesser amounts of the radioisotope. Since virtually no grains appear over the erythrocytes, they must contain comparatively little ^{65}Zn. No difference between the accumulation of radiozinc in nucleus and cytoplasm of the leucocyte can be discerned.

^{65}Zn concentration by the prostate

Among the organs assayed at autopsy, the prostate holds intermediate rank with respect to its ability to concentrate ^{65}Zn (Table II). For the eight males so studied, the mean ^{65}Zn concentration is 0·00495 per cent dose/g. (wet weight)

with values ranging between 0·00150 and 0·0117. Compared to the other organs, the ability of the gland to take up administered ^{65}Zn is very variable; it is also not related to time.

This finding of widely scattered radiozinc values independent of the interval following the infusion of the tracer is reinforced by the measurements made on the surgical specimens of the prostate obtained from 36 patients (Table III). For all 44 male patients, the concentrations ranged between 0·00150 and 0·0315

EXPLANATION OF PLATES

FIG. 1–5.—Patient M. K—. Radioautograph of bone marrow smear, prepared 8 days following administration of 100 μc ^{65}Zn, 215-day exposure. Giemsa.

FIG. 1.—Note large number of grains over early forms, fewer over leucocytes and virtual absence of grains over erythrocytes. ×180.

FIG. 2.—Megakaryocyte demonstrating marked ^{65}Zn localization. ×405.

FIG. 3.—Normoblast showing very high concentration of ^{65}Zn. ×405.

FIG. 4.—Myelocyte indicating considerable uptake of radiozinc. ×405.

FIG. 5.—Note moderate ^{65}Zn uptake in the 4 neutrophils. ×405.

FIG. 6.—Patient F. S—. Radioautograph of bone marrow smear prepared 6 days following administration of 50 μc ^{65}Zn. The metamyelocyte is heavily labeled with ^{65}Zn. 205-day exposure. Giemsa. ×405.

FIG. 7.—Patient M. F— (benign prostatic hyperplasia). Radioautograph of prostatic section from surgical specimen obtained 7 days after administration of 50 μc ^{65}Zn. Distribution of the radioisotope is spotty and largely confined to the border epithelial cells of the smaller acini. 61-day exposure. Methyl green-pyronin. ×42.

FIG. 8.—Patient L. E— (benign prostatic hyperplasia). Radioautograph of prostatic section from surgical specimen obtained 21 days after administration of 50 μc ^{65}Zn. Parts of several small acini are labeled with radiozinc. 119-day exposure. Methyl green-pyronin. ×42.

FIG. 9.—M. F— (same as Fig. 7—different section). Maximum ^{65}Zn localization is along the luminal border. The prostatic concretions are not as heavily labeled. 136-day exposure. Methyl green-pyronin. ×42.

FIG. 10.—Patient L. E— (same as Fig. 8). One of three adjacent follicles is very actively concentrating the radioisotope. The surrounding stroma does not pick up ^{65}Zn appreciably. Photographic grains appear principally over the border epithelium. × 180.

FIG. 11.—Patient C. L— (benign prostatic hyperplasia). Radioautograph of prostatic section from surgical specimen obtained 49 days after administration of 50 μc ^{65}Zn. Uptake by the follicle is not uniform. 65-day exposure. H. and E. ×180.

FIG. 12.—Patient L. E— (same as Fig. 8—different section). Variable distribution of ^{65}Zn in border epithelium of acinus. Methyl green-pyronin. ×405.

FIG. 13.—Patient L. E— (same as Fig. 8). Microscope focused on photographic grains over luminal border. ×405.

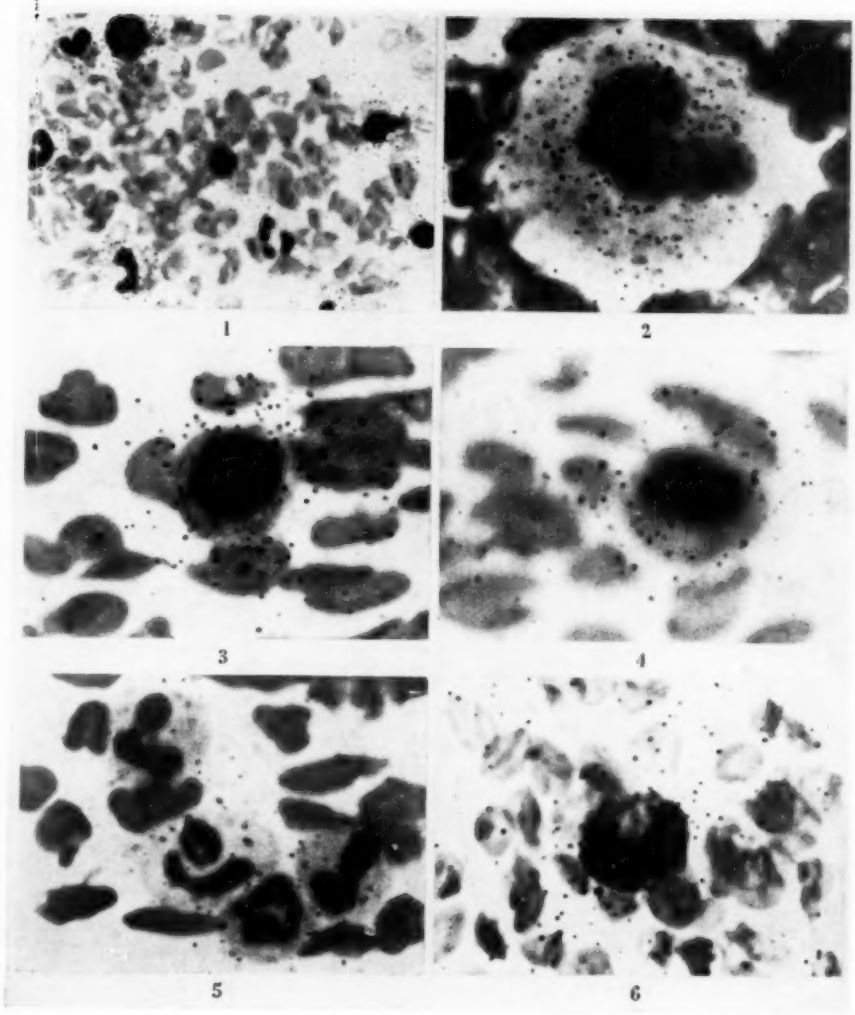
FIG. 14.—Patient L. E—. Same field and magnification as Fig. 13, focused on photographic grains over lumen.

FIG. 15.—Patient C. L— (same as Fig. 11). Sequestered cellular material in the holocrine type acinus is labeled as well as the border epithelial cells of this and the neighboring follicle. ×180.

FIG. 16.—Patient M. F— (same as Fig. 7). ^{65}Zn is taken up predominantly by the cytoplasm of the border epithelial cells. Relatively few grains appear over the adjacent stroma. ×405.

FIG. 17.—Patient M. F— (same as Fig. 7). Another field demonstrating the localization of ^{65}Zn in the cytoplasm of the acinar epithelium. Lesser amounts of tagged material are found in the lumen. ×405.

FIG. 18.—Patient E. Mc— (lymphoma in prostate). Radioautograph of prostatic section from necropsy specimen obtained 9 days after administration of 50 μc ^{65}Zn. Left; focus is on grains; right: focus is on the plane of the cells. Note that the radioisotope is generally outside the nucleus. 47-day exposure. Methyl green-pyronin. ×405.



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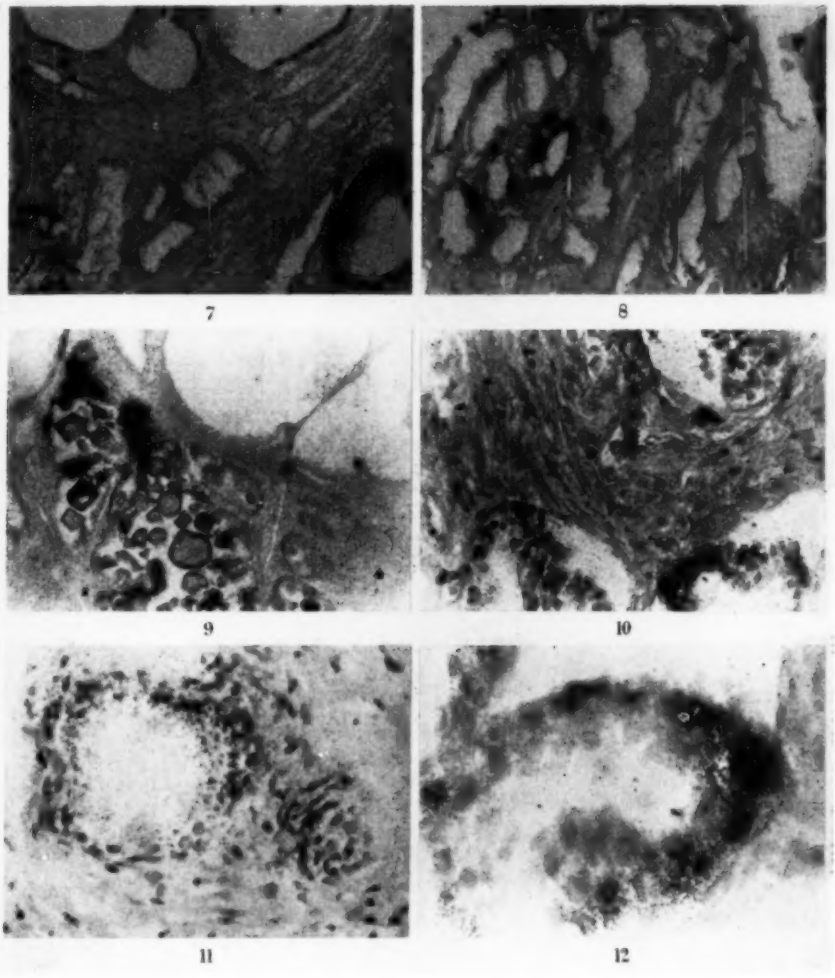

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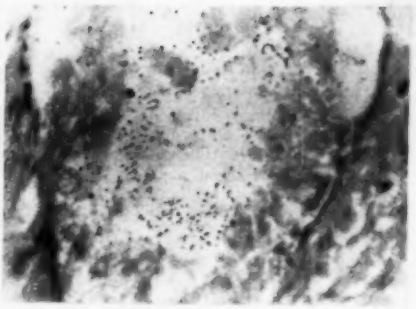

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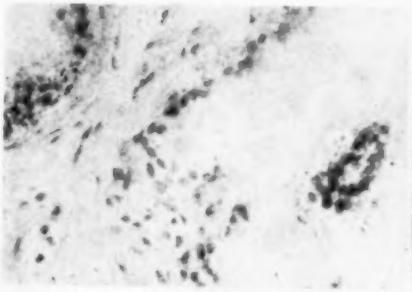

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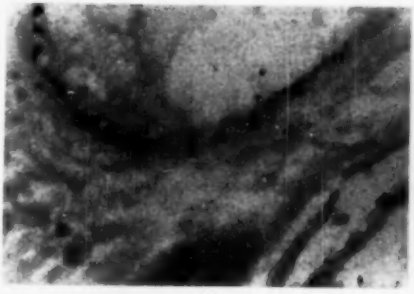

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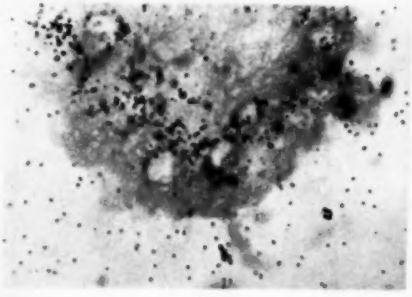

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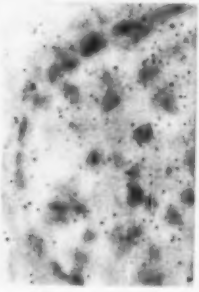

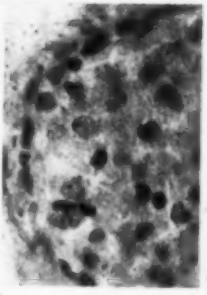

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TABLE II.—^{65}Zn *Concentration (per cent dose/g. wet weight* $\times 10^{-3}$) *in Various Organs of* 14 *Patients at Autopsy*

Patient	Sex	Age	Time from dose (days)	Diagnosis	Liver	Pancreas	Spleen	Prostate	Seminal vesicles	Lung	Bladder	Skeletal muscle
J. W—	♀	72	1	Carcinoma of breast with metastases	24·0	7·20	—	—	—	2·50	1·50	0·820
J. F—	♀	34	1	Carcinoma of colon	28·1	5·29	6·78	—	—	1·32	0·975	1·39
G. C—	♂	70	2	Laryngeal carcinoma; benign prostatic hyperplasia	49·4	23·0	6·14	3·26	—	3·20	2·62	4·30
R. S—	♂	32	3	Hemangiosarcoma; normal prostate	33·6	16·6	12·5	4·46	3·51	3·78	3·57	1·87
F. W—	♀	52	3	Carcinoma of colon with metastases	20·5	16·6	10·3	—	—	2·90	1·59	1·79
J. C—	♀	72	5	Ovarian carcinoma	39·0	6·10	—	—	—	2·20	1·80	1·20
F. S—	♂	60	7	Hodgkin's disease; normal prostate	43·0	7·70	—	3·13	3·00	2·10	2·30	3·60
E. M—	♂	52	8	Carcinoma of lung with metastases; lymphoma in prostate	23·2	3·29	—	6·30	—	1·56	0·973	1·52
E. A—	♂	58	9	Carcinoma of lung with metastases; benign prostatic hyperplasia	22·2	—	4·17	2·77	3·26	2·10	2·16	2·01
I. F—	♀	70	12	Carcinoma of lung	29·2	10·1	10·1	—	—	2·84	4·02	2·14
J. Mi—	♀	41	20	Carcinoma of breast with metastases	5·24	3·71	2·07	—	—	2·20	2·98	0·845
J. M—	♂	56	60	Carcinoma of lung with metastases; benign prostatic hyperplasia	5·90	—	—	11·7	—	—	2·40	4·40
G. S—	♂	49	81	Esophageal carcinoma; normal prostate	12·7	2·77	—	5·60	1·76	1·19	2·10	1·88
F. L—	♂	58	174	Hypernephroma with lung metastases; benign prostatic hyperplasia	2·70	0·330	—	1·50	1·40	0·810	1·00	0·220

TABLE III.—*^{65}Zn Concentration (per cent dose/g. wet weight $\times 10^{-3}$) in the Prostates of 44 Patients*

Patient	Age	Time from dose (days)	^{65}Zn concentration
A. Normal Prostate			
R. S—	32	3	4·46
J. N—	72	6	10·2
F. S—	60	7	3·13
G. S—	49	81	5·60
Average 5·85 ± 2·66*.			
B. Benign Prostatic Hyperplasia			
B. R—	83	1	10·3
S. F—	76	1	5·21
G. C—	70	2	3·26
J. G—	75	3	21·2
W. O'G—	64	3	6·23
L. H—	71	4	8·23
M. P—	72	5	14·9
B. B—	58	5	14·7
G. P—	66	5	9·87
W. G—	65	5	15·1
J. C—	72	6	22·9
H. S—	82	6	5·82
M. F—	75	7	17·1
B. C—	76	7	8·59
G. M—	75	7	9·37
J. D—	72	8	6·89
J. S—	82	8	6·50
E. A—	58	9	2·77
M. G—	74	9	13·9
S. G—	53	11	1·87
F. K—	75	11	12·3
J. Sc—	73	11	6·05
S. K—	84	13	10·1
J. St—	70	14	19·7
M. Gz—	77	18	4·96
I. Le—	68	19	17·1
H. F—	73	20	10·9
L. E—	72	21	10·3
M. C—	85	21	12·9
M. T—	71	23	31·5
A. W—	76	26	10·4
C. L—	74	49	5·60
J. Mc—	56	60	11·7
F. L—	58	174	1·50
I. L—	72	196	4·12
Average 10·78 ± 6·41*.			
C. Prostatic Malignancy			
J. C—	63	3	2·44
A. P—	82	4	13·7
H. A—	62	8	6·89
L. B—	85	8	6·50
E. M—	52	8	6·30
Average 7·17 ± 3·63*			

* Standard deviation

per cent dose/g. during the 196 day period involved. This 20-fold variation in ^{65}Zn concentration among glands was the impetus for investigating the distribution of the radioisotope within the prostate.

Determinations were made of the ^{65}Zn concentrations in different portions of the prostate obtained from each of 15 patients undergoing surgery for benign prostatic hypertrophy (Table IV). The fluctuation in ^{65}Zn values is very great, as much as a seven-fold range being observed for one case. For 12 of these patients, the coefficient of variation for the concentration within the gland was greater than 20 per cent. Moreover, levels of the radioisotope cannot be related consistently to specific anatomical regions of the gland.

TABLE IV.—*Variation in ^{65}Zn Concentration (per cent dose/g. wet weight $\times 10^{-3}$) Within the Hyperplastic Prostate of 15 Patients*

Patient	Age	Time from dose (days)	Lobe (number of samples)	Concentration	Mean concentration	Coefficient of variation
S. F—	76	1	Right (1)	5·04	5·21	8·72
			Left (1)	5·27		
			Median (1)	4·65		
			Anterior (1)	5·89		
J. G—	75	3	Right (8)	11·7, 14·3, 14·3, 16·0, 18·8, 22·1, 35·3, 37·4	21·2	43·6
L. H—	71	4	Not identified (6)	6·96, 7·45, 7·73, 8·34, 9·00, 9·85	8·23	11·0
M. P—	72	5	Right (4)	9·32, 15·1, 17·1, 18·2	14·9	22·9
G. P—	66	5	Left (8)	6·20, 6·40, 7·37, 8·67, 10·7, 11·2, 12·9, 15·5	9·87	31·3
B. B—	58	5	Right (3)	4·38, 10·4, 30·0,	14·7	53·9
			Left (2)	14·9, 21·6		
			Median (2)	10·2, 11·5		
J. N—	72	6	Right (1)	17·7	10·2	46·2
			Left (1)	8·05		
			Median (1)	4·91		
			Anterior (1)	10·3		
H. S—	82	6	Right (2)	9·02, 12·0	5·82	54·3
			Left (2)	5·71, 8·93		
			Posterior (2)	4·58, 5·45		
J. D—	72	8	Right (1)	16·0	10·5	52·7
			Left (1)	5·08		
F. K—	75	11	Right (1)	13·0	12·3	26·5
			Left (1)	15·6		
			Anterior (1)	13·6		
			Posterior (1)	6·89		
J. Sc—	71	11	Not identified (4)	2·03, 3·58, 6·59, 12·0	6·05	63·8
J. St—	70	14	Right (2)	12·2, 32·8	19·7	45·5
			Left (2)	10·6, 23·9		
M. C—	85	21	Right (8)	7·81, 9·52, 10·7, 11·2, 13·8, 14·6, 17·6, 18·1,	12·9	27·1
M. T—	71	23	Not identified (3)	28·1, 32·1, 34·4	31·5	9·46
I. L—	72	196	Right (1)	5·29	4·12	29·8
			Left (1)	4·66		
			Median (1)	2·41		

No significant difference in the concentration of ^{65}Zn can be associated with the condition of the prostate. The results for the entire 44 patients studied are assembled in Table III, grouped according to the status of the gland. For the four patients with normal prostates, a mean ^{65}Zn concentration of $5{\cdot}85 \pm 2{\cdot}66^* \times 10^{-3}$ per cent dose/g. was found; the corresponding average for the five patients having prostatic malignancy is $7{\cdot}17 \pm 3{\cdot}63 \times 10^{-3}$ per cent dose/g. These levels are lower, but not significantly, than those for the 35 patients having benign hyperplasia of the gland (average $10{\cdot}78 \pm 6{\cdot}41 \times 10^{-3}$ per cent dose/g.). Since a valid statistical difference is not demonstrable, these 44 patients are considered a homogenous population having a mean ^{65}Zn concentration of $9{\cdot}92 \pm 6{\cdot}15 \times 10^{-3}$ per cent dose/g.

These marked differences in ^{65}Zn concentrations within the prostate are encountered also on the histological level as demonstrated by radioautography (Fig. 7–18). While the follicles have a great affinity for the radioisotope, they exhibit vast differences in this respect. Our preparations indicate that the follicular deposition of ^{65}Zn is spotty. The glandular units frequently involved in localizing the radioisotope are the smaller ones. Although follicular uptake varies, both apocrine- and holocrine-like units actively accumulate the radiometal. The sequestered cellular material within the holocrine type acini is strongly labeled. Grains are frequently located over the lumens and ducts, although never as many as over the border epithelium of the acini. For active follicles, ^{65}Zn is restricted to the border epithelial cells, principally the cytoplasm. No significant amounts of activity are present in adjacent muscle and connective tissues.

The attempts to perform *in vivo* surveys proved unsatisfactory. While the detectors indicated higher than background levels when inserted into the rectum, the counting pattern did not correspond to the anatomical location of the gland.

DISCUSSION

Little is available in the literature regarding the fate of this radioisotope, particularly its behaviour in man. Adequate studies of the fraction of the tagged zinc concentrated in various organs by an appreciable number of human subjects are yet to be reported. It is consequently difficult to relate our findings with those published by others.

^{65}Zn as a tracer.—A question meriting evaluation is whether the ^{65}Zn doses employed by us, as well as those given by others in conducting human and animal studies, are physiological. The relationship of the specific activity of administered ^{65}Zn to organ localization requires clarification. This consideration may have a strong bearing on the discrepancies found between the distribution of radiozinc and that of stable zinc. For laboratory animals, whose body weights and total zinc contents are much smaller than those of man, it may well be that the amount of carrier given has influenced the observed behaviour of ^{65}Zn. Since figures for specific activity are frequently omitted, the importance of carrier cannot be assessed for many reports (Tables V and VI).

The ^{65}Zn furnished us has a specific activity ranging between 83·6 and 93·5 mc/g., so that about one-half to one milligram of zinc was contained in each dose. These amounts of stable zince are small compared to the total body content of

* Standard deviation.

man, approximately 2 grams (Widdowson, McCance and Spray, 1951). The ingestion of milligram quantities of carrier zinc, corresponding to about one-tenth of the daily intake of the element (McCance and Widdowson, 1942), may be consequential. The simultaneous measurements of stable and radioactive zinc concentrations, as recommended by Vallee (1959), may not prove as valuable as anticipated unless higher specific activity or even carrier-free ^{65}Zn is employed.

Organ concentration of zinc and ^{65}Zn.—It is interesting to compare the stable zinc levels found in the various organs of man with the accumulation of ^{65}Zn in these organs of man, dog, rat and mouse. The available concentration data for both zinc nuclides have been collected in Table V. The entries have been arbitrarily expressed relative to the concentration in the liver to facilitate comparison and to permit inclusion of more of the published findings. Assuming the body contains 2·5 grams of the metal, the percentage of the total body zinc in a gram of each organ has been computed (Table V, column 2), a parameter somewhat analogous to the expression for concentration of the ^{65}Zn (per cent dose/g.).

The agreement between stable zinc and ^{65}Zn organ to liver concentration ratios for lung and spleen is good both for man and the laboratory animals; however, divergent values are encountered for prostate, pancreas and skeletal muscle. The stable zinc level of the prostate is nearly three times greater than that of the pancreas and about twice that of the liver. With respect to ^{65}Zn, however, localization by the prostate is similar to that of the pancreas and about half as much as by the liver. These results are in sharp contrast to the marked affinity for ^{65}Zn by the dorsolateral prostate of the rat, which takes up almost 11 times as much as the liver (Gunn *et al.*, 1955). While for man, stable zinc and radiozinc concentrations in the pancreas are lower than the corresponding liver values, higher ^{65}Zn ratios of pancreas to liver are reported for the experimental animals. Thus, it would seem that ^{65}Zn concentrations in the prostate and pancreas of animals are not indicative of the corresponding behaviour of stable zinc in these organs of man.

Total zinc and ^{65}Zn in organs.—A knowledge of the total stable zinc content of various organs as well as the corresponding values for ^{65}Zn would be of significance. Making the assumption that the stable metal and radiometal are distributed uniformly throughout the organ, an estimate of the respective amounts present can be obtained from their concentrations and the weights of the organs (Table VI). The validity of this assumption is open to question, particularly in view of the great variation in ^{65}Zn concentration found within the prostate (Table IV). This approach has the overriding merit, however, of providing a basis for a crude analysis of ^{65}Zn kinetics.

Considering the errors made in assuming homogeneous organ distribution of ^{65}Zn and in employing average organ weights, the agreement is surprisingly good between both forms of zinc for most organs, including the prostate and pancreas (Table VI). Organ uptake of ^{65}Zn, unlike concentration measurements, could be thus expected to reflect the handling of stable zinc. The liver, however, is a notable exception, since it takes up a much higher fraction of the ^{65}Zn dose than its relative content of the stable metal.

The various organs of man and laboratory animals exhibit differences in their uptake of ^{65}Zn. While as much as 65 per cent of the ^{65}Zn dose is picked up by the liver in man, hardly one per cent localizes within the prostate gland, the lowest of the organs studied. Muscle, because of its huge mass, has the

TABLE V.—*Stable Zinc and ^{65}Zn Concentrations in Man, Dog, Mouse and Rat*

Organ	Stable zinc, Man: Conc. (γ/g. wet*)	Stable zinc, Man: Organ conc. × 100 / Total body zinc†	Stable zinc, Man: Organ / Liver
Liver	101 –13·9	$4·04–0·556 \times 10^{-2}$	1·00–1·00
Prostate	220 –16·5	8·80–0·0660	2·18–1·19
Skeletal muscle	30‡	1·20	0·30
Pancreas	80·3–10·4	3·21–0·416	0·794–0·748
Spleen	36·4–9·36	1·46–0·374	0·362–0·672
Lung	34·1–7·26	1·36–0·290	0·337–0·521
Remarks	* Wet weights used throught table. † 2·5 g. total body zinc assumed. ‡ Vallee (1959).		
Reference	Calculated from data of Tipton (1960).		

Organ	Radioactive zinc (^{65}Zn), Man: Conc. (% dose/g.)	Organ / Liver	Conc. (% dose/g.)	Organ / Liver	Organ / Liver
Liver	$49·4–2·70 \times 10^{-3}$	1·00–1·00	$40·5 \times 10^{-3}$	1·00	1·00
Prostate	31·5–1·50	0·638–0·557	28·0–2·34	0·690–0·058	—
Skeletal muscle	4·40–0·220	0·089–0·082	3·57	0·088	—
Pancreas	23·0–0·330	0·467–0·122	18·2	0·450	0·21
Spleen	12·5–2·07	0·253–0·766	7·15	0·176	—
Lung	3·78–0·810	0·077–0·300	—	—	—
^{65}Zn dose	50–100 μc ; s.a.¶ : 83·6–93·5 mc/g.		50–100 μc ; s.a. : 50–100 mc/g.		Activity (μc) not stated ; 270–380 γ.
Remarks	Range observed for prostates (44 cases) over 196 days ; other organs (14 cases) over 174 days. ¶ Specific activity.		Range observed for prostates (11 cases) over 4 days ; other organs, 1 case at 8 days.		5 cases, between 9–23 days.
References	Montefiore Hospital.		Daniel *et al.* (1956).		Ross *et al.* (1958).

Organ	Radioactive zinc (^{65}Zn), Dog: Conc. (% dose/g.) 8 hr.	Conc. 170 hr.	Organ / Liver 8 hr.	Organ / Liver 170 hr.	Conc. (% dose/g.) 8 hr.	Organ / Liver 8 hr.	Mouse: Conc. (% dose/g.) 2 hr.	Conc. 170 hr.	Organ / Liver 2 hr.	Organ / Liver 170 hr.
Liver	34×10^{-2}	$2·50 \times 10^{-2}$	1·00	1·00	$14·8 \times 10^{-2}$	1·00	25	4·7	1·00	1·00
Prostate	—	—	—	—	—	—	—	—	—	—
Skeletal muscle	0·6	1·8	0·02	0·72	—	—	1·4	1·2	0·66	0·26
Pancreas	28	6·8	0·82	2·72	15·5	1·05	43	4·9	1·72	1·04
Spleen	10	3·8	0·29	1·52	3·1	0·21	12·5	2·5	0·50	0·53
Lung	3·4	2·7	0·10	1·10	—	—	8·0	2·0	0·32	0·43
^{65}Zn dose	Activity (μc) not stated ; 6·52–8·15 γ.				100 μc ; s.a. not stated.		Activity (μc) not stated; 0·33–1·6 γ.			
References	Sheline *et al.* (1943).				Meschan *et al.* (1959).		Sheline *et al.* (1943).			

TABLE V—*contd.*

	Radioactive zinc (^{65}Zn) Rat									
	Conc. (% dose/g.)		Organ Liver		Conc. (% dose/g.)		Organ Liver		Organ Liver	
	4 hr.	92 hr.	4 hr.	92 hr.	3 hr.	10 d.	3 hr.	10 d.	24 hr.	48 hr.
Liver	1·77	0·75	1·00	1·00	3·91	0·34	1·00	1·00	1·00	1·00
Prostate	—	—	—	—	—	—	—	—	8·25§	10·7§
									0·50	0·53
Skeletal muscle	0·07	0·19	0·04	0·25	0·14	0·14	0·04	0·41	1·85	1·00
Pancreas	3·11	0·55	1·76	0·73	4·84	0·28	1·24	0·83	—	—
Spleen	0·62	0·44	0·35	0·59	1·88	0·21	0·48	0·62	—	—
Lung	—	—	—	—	0·92	0·19	0·24	0·56	—	—
^{65}Zn dose	75–200 μc; s.a.: 370–520 mc/g.				5 μc; s.a. not stated.				0·4 mc/kg.; s.a.: 120–164 mc/g.	
Remarks									§ Dorsolateral prostate. Ventral prostate.	
References	McIsaac (1955).				Ballou (1959).				Gunn *et al.* (1955).	

maximum uptake. It is interesting to note that both the spleen (about 6 per cent) and the lung (about 5 per cent) take up more than the pancreas (2·7 per cent), an organ once believed to be very much concerned with zinc metabolism. Our figures are in fair accord with the scanty data available pertaining to man (Daniel *et al.*, 1956; Prout *et al.*, 1959; Ross *et al.*, 1958) and the dog (Sheline *et al.*, 1943; Meschan *et al.*, 1959), but are higher than the corresponding values for the mouse (Sheline *et al.*, 1943) and rat (Gilbert and Taylor, 1956; Ballou, 1959).

The variation of ^{65}Zn uptake in the liver with time is noteworthy (Fig. 19). The maximum level occurs about the seventh day, declining quite slowly thereafter. As late as 174 days after injection, 3·8 per cent of the dose is still present in the liver. In comparison to the mouse and dog, the levels and turnover of ^{65}Zn in the liver of man are quite different (Fig. 19). The peaks for the animals are reached early, before the end of the first day. Furthermore, the disappearance of ^{65}Zn in the mouse and dog is so rapid, that by the end of the first week, only 3·3 per cent of the dose remains in the liver of the mouse and 3·5 per cent in the same organ of the dog. Gilbert and Taylor (1956) have also observed an early fall of ^{65}Zn uptake in the liver of the rat, so that only 1·6 per cent of the dose is retained 35 days after administration of the dose.

As the determinations of permissible levels of ^{65}Zn in man have been hitherto based on animal studies (National Bureau of Standards, 1959), the values and the turnover being reported here may have a bearing on these calculations. Bone is being taken as the critical organ, since the studies in animals conducted by Sheline *et al.*, (1943) indicate a longer biological half-life of administered ^{65}Zn in bone than either in liver or in pancreas. As noted earlier, while both of the latter organs in man accumulate high levels of ^{65}Zn rapidly, the turnover in the pancreas is faster. Our data indicate that the biological half-life of ^{65}Zn in the

liver of man is about 75 days, a gross estimate because of the few points available (Fig. 19). Although considerable error is associated with this value, it is certainly longer than the 23 days found for bone of the dog (Sheline *et al.*, 1943). Further studies along these lines are needed to establish more precisely the turnover in human bone and liver in order to reduce the uncertainty in dosage computations.

TABLE VI.—*Stable Zinc and* ^{65}Zn *Uptake (per cent dose/organ) in Man, Dog, Mouse and Rat*

	Stable zinc			
	Man			
Organ	Assumed organ wt. (g.)	Organ zinc (g.)	Organ zinc / Body zinc* × 100	Organ / Liver
Liver	2,000	0·202–0·0278	8·08–1·11	1·00–1·00
Prostate	30	0·06660–0·00050	0·264–0·0200	0·033–0·018
Skeletal muscle†	30,000	0·900‡	36·0	4·45–32·7
Pancreas	100	0·00803–0·00104	0·321–0·0416	0·040–0·038
Spleen	200	0·00728–0·00187	0·291–0·0748	0·363–0·067
Lung	1,000	0·0341–0·00726	1·36–0·290	0·168–0·261
References	Calculated from data of Tipton (1969).			

	Radioactive zinc (^{65}Zn)					
	Man					
		Organ		Organ		Organ
Organ	Uptake	Liver	Uptake	Liver	Uptake	Liver
Liver	65·5–3·78	1·00–1·00	81	1·00	35·6	1·00
Prostate	0·945–0·033	0·014–0·009	0·840–0·058	0·010–0·001	—	—
Skeletal muscle†	92·5–5·81	1·41–1·54	85	1·05	—	—
Pancreas	2·67–0·033	0·041–0·099	1·82	0·023	0·69	0·019
Spleen	5·86–0·644	0·090–0·170	1·43	0·018	—	—
Lung	4·94–0·972	0·076–0·257	—	—	—	—
References	Montefiore Hospital.		Daniel *et al.* (1956).		Ross *et al.* (1958).	

	Radioactive zinc (^{65}Zn)											
	Dog						Mouse				Rat	
	Uptake		Organ Liver		Uptake	Organ Liver	Uptake		Organ Liver		Uptake	Organ Liver
Organ	8 hr.	170 hr.	8 hr.	170 hr.	8 hr.	8 hr.	2 hr.	170 hr.	2 hr.	170 hr.	35 d.	35 d.
Liver	34	3·5	1·00	1·00	87·3	1·00	25	3·3	1·00	1·00	1·6	1·00
Prostate	—	—	—	—	—	—	—	—	—	—	—	—
Skeletal muscle†	9·6	36	0·28	10·3	—	—	—	—	—	—	9·5	5·9
Pancreas	3·1	0·69	0·09	0·20	4·1	0·05	1·7	0·44	0·07	0·13	—	—
Spleen	0·93	0·31	0·03	0·09	4·4	0·05	1·3	0·14	0·05	0·04	0·1	0·06
Lung	0·73	0·71	0·02	0·20	—	—	1·1	0·26	0·04	0·08	—	—
References	Sheline *et al.* (1943).				Meschan *et al.* (1959).		Sheline *et al.* (1943).				Gilbert and Taylor (1956).§	

* 2·5 g. total body zinc assumed.
† Assuming 41 per cent body weight is muscle.
‡ Calculated, based on value in Vallee (1959).
§ 2 μc ^{65}Zn, specific activity : 8 curies/g. ; all other ^{65}Zn doses indicated in Table V.

^{65}Zn in bone marrow.—The high uptake of ^{65}Zn by leucocytes as compared to erythrocytes demonstrated by our radioautographs confirm observations based on studies with stable zinc and recently with ^{65}Zn. Thus, it is believed that the leucocyte probably contains more zinc than any other cell of the body (Vikbladh, 1951); the white cells have as much as 3 per cent of whole blood zinc (Vallee and Gibson, 1948). The zinc ratio of leucocyte to erythrocyte is about 20 (Ross *et al.*, 1958) or 15 (Dennes, Tupper and Wormall, 1961). This latter group has found also that two hours after incubation of whole blood with a ^{65}Zn-glycine solution, the leucocyte to erythrocyte ratio for the radioisotope is about 57. Of more direct interest are their experiments in rabbits, indicating that 24 hours

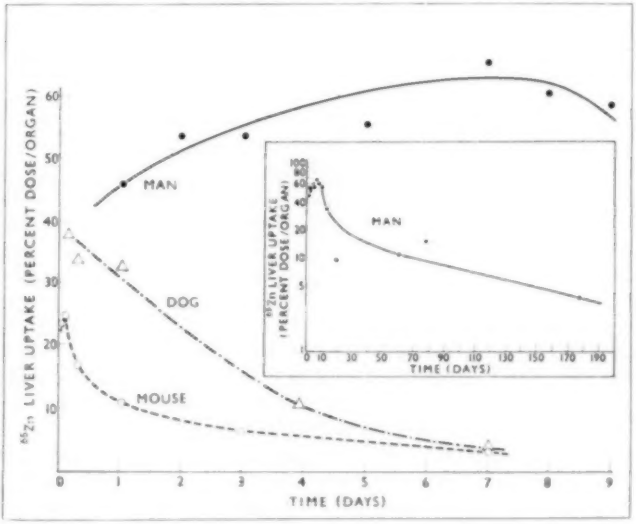

FIG. 19.—^{65}Zn liver uptake for man compared to the values reported by Sheline *et al.* (1943) for dog and mouse. Each point plotted for the mouse refers to the average of 3–7 animals. For man and for dog, each point represents the uptake of a single subject. *Inset.*—The long-term variation of ^{65}Zn uptake by the human liver is graphed semi-logarithmically.

after administration of ^{65}Zn, a similarly high ratio (about 30) is found in the bone marrow.

^{65}Zn in the prostate.—A dominant feature of the findings presented here is the low and variable total uptake of ^{65}Zn by the human prostate (Table IV). As already remarked, hardly one per cent of the administered radioisotope is taken up by the gland, an approximate value in view of the non-uniform ^{65}Zn concentration within the organ. In any event, ^{65}Zn in the prostate of man does not approach the concentration and uptake of ^{131}I by the thyroid, as had been claimed (Gunn *et al.*, 1955).

The fluctuation of prostatic ^{65}Zn concentration among our patients is considerably greater than that reported by others. While an eight-fold variation was found in the necropsy specimens from eight male patients, an even greater ratio of about 15 was encountered in the surgical specimens from 36 patients

(Table III). A factor of only 12 is indicated in the series of 15 patients studied with ^{65}Zn by Daniel *et al.* (1956) and an even smaller ratio, about seven, is found for the 21 cases investigated by Prout *et al.* (1959). It should be noted that stable zinc levels are likewise variable, Hoare, Delory and Penner (1956) having observed an 11-fold range in 19 normal prostates.

Our observation that the variation of ^{65}Zn concentration within the gland is unrelated to anatomy (Table IV) does not agree with some stable zinc determinations in man or with radiozinc studies in the rat. Thus, Kerr, Kerestechi and Mayoh (1960) concluded that levels of stable zinc increase toward the apex of the human prostate. They found great variations in concentration of the metal, as much as 18-fold, for different portions of the gland. On the other hand, Mager, McNary and Lionetti (1953), using histochemical methods, could not detect differences in zinc content between the anterior and posterior lobes of the human, canine and simian prostates. In the rat, Gunn *et al.* (1955) found that the dorsolateral prostate concentrates about 20 times as much ^{65}Zn as the ventral portion. More recently, they have shown that the lateral tip of the dorsolateral gland, composed exclusively of apocrine type acini, is concerned with ^{65}Zn uptake, but is low in fructose; the dorsal tip, however, comprised of holocrine-like units, is devoid of ^{65}Zn, but is high in fructose (Gunn and Gould, 1957).

The low uptake of ^{65}Zn by the prostate together with the relatively high levels of the radioisotope in the liver, bladder and blood (Graig and Siegel, 1960) account, we believe, for our failure to perform satisfactory measurements of uptake by the prostate using rectal detectors. This comparatively poor localization dims the hope of applying such *in vivo* counting techniques for diagnosis or for determining indices of function, unless much higher ratios are encountered for pathological states of the prostate. Our findings are in accord with the meager evidence available (Mawson and Fischer, 1952; Daniel *et al.*, 1956; Prout *et al.*, 1959; Kerr *et al.*, 1960) which suggests that the reverse is more probable.

Neither ^{65}Zn concentration nor total uptake by the prostate was found to vary with the period elapsing between administration of the radioisotope and removal of the specimen, a disturbing finding. This is obviously not a firm conclusion, since it is based on single observations for each patient. The marked variation in ^{65}Zn concentration, the error made in estimating the weight of the gland, and the unknown role of carrier already alluded to, are additional factors obscuring any temporal relationship, if indeed one exists.

^{65}Zn concentration and prostatic status.—We were unable to demonstrate a correlation between the concentration of the tracer and the state of the prostate. Such a relationship might be expected in view of the stable zinc determinations indicating depressed concentrations in the presence of malignancy (Mawson and Fischer, 1952; Hoare *et al.*, 1956; Kerr *et al.*, 1960). While the mean ^{65}Zn concentration of the five malignant prostates in our series is higher than the average for the four normal glands and lower than that of the 35 benign hyperplastic prostates, the differences are not statistically significant due to the very great dispersion among the individual values. Among 15 patients investigated, Daniel *et al.* (1956) found that the ^{65}Zn concentrations in two of their three malignant prostates were not appreciably lower. Indeed, the highest ^{65}Zn level in any tissue studied by them occurred in the untreated prostatic carcinoma of their third patient. Prout *et al.* (1959) encountered in only one of 12 malignant prostates a markedly lower concentration of the tracer than in adjacent normal tissues.

Additional studies involving larger series of patients are still required to establish whether a relationship does actually exist between concentration of the tracer by the gland and its pathology.

Age and ^{65}Zn concentration in prostate.—An interesting question is raised as a result of arranging the ^{65}Zn concentration in the prostate according to the age of the patient (Table VII). Does the concentration of ^{65}Zn rise with age, attaining maximum values in the seventies, to decline thereafter? The variation in the concentration data precludes a definitive response, but further inquiry seems warranted. It is noteworthy that this trend of increasing concentration with age is present also in the data of Prout *et al.* (1959) for their nine patients with benign hyperplasia. Perhaps this rise in ^{65}Zn with age is related to the corresponding increase in weight of the prostate and its decrease sometime during the eighth decade (Simmonds, 1918). In this connection, mention should be made of the finding of Fischer *et al.* (1955) that the stable zinc concentration of the dorsolateral prostate of the rat increases with age, reaching a maximum at about 160 days. Gunn *et al.* (1955) also found that the prostates of rats six months and older picked up the largest amounts of ^{65}Zn.

TABLE VII.—*^{65}Zn Concentration (per cent dose/g. wet weight $\times 10^{-3}$) in the Prostates of 44 Patients as a Function of Age*

Decade	Number of patients	Average	Range
30–39	1	4·46	—
40–49	1	5·60	—
50–59	6	6·91	1·87–14·7
60–69	7	10·82	2·44–21·2
70–79	22	11·71	3·26–31·5
80–89	7	9·40	5·82–13·7

Cellular and intracellular ^{65}Zn in the prostate.—The wide fluctuation in ^{65}Zn content in the prostate gland, determined grossly by sample counting, is also found on a histological level by radioautography, which demonstrates that the follicles differ in their affinity for ^{65}Zn. Histochemical confirmation is provided by the studies performed with dithizone staining of the prostates of developing rats (Fischer *et al.*, 1955). As the rats become able to breed (about 50 days), a few areas within isolated acini become stainable. Several days thereafter, scattered acini are seen in which all the cells react positively. Spotty activity of follicles has been encountered in the study of other glands, an outstanding example being the thyroid gland with respect to its variable localization of ^{131}I.

Some of the other findings from our radioautographs are also in good agreement with the literature. The localization of ^{65}Zn by the border epithelium is in accord with the radioautograph published by Daniel *et al.* (1956) for human prostate and with those presented by Wetterdal (1958) and Millar, Vincent and Mawson (1961) for the dorsolateral prostate of the rat. Using the dithizone stain, Mager *et al.* (1953) studied the prostates of the dog, man, monkey and rabbit, reporting that the apical portions of the secretory cells were most intensely stained. They found that the associated connective and muscle tissues did not contain zinc. Employing a dithizone complex-forming solution to enhance specificity for zinc, this group showed, also, that the nuclei were non-staining.

Fleischhauer (1957) has also observed stainable zinc in the apical border of prostatic cells in the rat, but the distal portions of the epithelium concentrated the metal only slightly; the periphery of the nucleus was occasionally positive for zinc. Gunn and Gould (1956) published a photomicrograph which demonstrated positive staining of the acini at the luminal border in the lateral prostate of the rat. Rixon and Whitfield (1959) confirmed this high concentration of zinc in lateral portions of the dorsolateral prostate of the rat, finding three regions with positive staining: apical cytoplasm, nucleoli and sub-epithelial stroma.

In spite of the evidence just cited and confirmed by our radioautographs, the observation that the nuclei of the apical epithelium contain little or no ^{65}Zn is unexpected, in view of the distribution of stable zinc in animal tumours and livers. Heath (1949) and Heath and Liquier-Milward (1950) have reported that zinc is associated with nucleoprotein from tumour tissues. Zinc has been found also in the nuclear fractions of normal rat livers (Thiers and Vallee, 1957) and of mouse mammary tumours and livers (Bartholomew, Tupper and Wormall, 1959). It should be remarked, however, that Rosenfeld and Tobias (1951) were unable to detect nucleoprotein-bound zinc in their studies.

The radioautographs indicate that both holocrine and apocrine types of follicles can actively pick up ^{65}Zn. As already noted, Gunn and Gould (1957) have shown that the lateral tips of the dorsolateral prostate, which are concerned with the localization of the radioisotope and devoid of fructose, are comprised exclusively of apocrine type acini. Conversely, the dorsal tips, which have a higher fructose content and reduced ^{65}Zn levels, have only holocrine units. The situation in man is, apparently, unlike that which prevails in the rat.

SUMMARY

The distribution of ^{65}Zn was studied in the organs of 14 patients at autopsy, occurring between 1 and 174 days following administration of 100 μc. The greatest concentration of the radioisotope is reached within a few days in the liver (about 0·050 per cent dose/g. wet weight), which localizes about 2–8 times more than the pancreas and as much as 10–30 times more than muscle. ^{65}Zn leaves the liver slowly, 0·013 per cent dose/g. still being present 81 days after infusion. A more rapid turnover of the administered radioisotope occurs in the pancreas. In this organ, the maximum concentration (about 0·023 per cent dose/g.) is attained by the second day; within a week, the level of the tagged metal has fallen by two-thirds, and by 81 days, hardly a tenth remains. The maximum concentration of ^{65}Zn in the spleen (0·013 per cent dose/g.) is about half that of the pancreas. For the spleen, as well as for those organs having even lower levels of the radioisotope (seminal vesicles, lung, bladder and muscle), no temporal relationship could be discerned.

The prostatic localization of ^{65}Zn was studied by assaying autopsy specimen from 8 of these patients and surgical specimens from 36 others undergoing surgery. For the entire series of 44 patients having normal, hyperplastic, or malignant prostates, the mean ^{65}Zn concentration is 0·00992 $\pm$ 0·00615 per cent dose/g., ranging between 0·0315 and 0·00150; these ^{65}Zn values are second only to those of the liver. No correlation could be demonstrated between the concentration of the radiometal and the condition of the prostate. By assaying samples of different lobes from the hyperplastic glands of 15 patients, marked variation in ^{65}Zn

concentrations is encountered within the organ unrelated to the anatomical subdivisions. Radioautographs of prostatic sections reveal that the deposition of the radioisotope, which is largely confined to the border epithelial cells, is not uniform. The smaller follicles accumulate the radioisotope predominantly, with both apocrine and holocrine type acini participating. Lesser amounts of the tagged metal are found in the lumens and ducts. ^{65}Zn is concentrated principally by the cytoplasm of these border epithelial cells, insignificant amounts being taken up by the nuclei and by the associated connective and muscle tissues.

Radioautographs, prepared from bone marrow aspirates obtained about one week after ^{65}Zn infusion, show that megakaryocytes and all early forms of the hematopoietic series are very heavily labeled. Mature leucocytes take up some ^{65}Zn, but comparatively little of the nuclide is present in the erythrocytes.

The distribution pattern of the radioisotope is discussed in relation to stable zinc determinations in man. Our ^{65}Zn data are compared also to those reported for experimental animals. Assuming uniform concentration throughout the organ, estimates are made of total organ uptake of ^{65}Zn. The maximum accumulation of the administered tracer occurs in skeletal muscle and in liver, while pancreas and prostate have by far the lowest uptake. The effect of some factors on prostatic levels of ^{65}Zn is considered. The intracellular deposition of ^{65}Zn by the prostate of man is compared to the histochemical studies of stable zinc in man and experimental animals.

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REFERENCES

Ballou, J. E.—(1959) 'Metabolism of Zn^{65} in the Rat'. Richland, Washington (Hanford Atomic Products Operation), HW-60062.

Bartholomew, M. E., Tupper, R. and Wormall, A.—(1959) *Biochem. J.*, **71**, 15.

Boyd, G. A.—(1955) 'Autoradiography in Biology and Medicine'. New York (Academic Press), p. 248.

Daniel, O., Haddad, F., Prout, G. and Whitmore, W. F.—(1956) *Brit. J. Urol.*, **28**, 271.

Dennes, E., Tupper, R. and Wormall, A.—(1961) *Biochem. J.*, **78**, 578.

Fischer, M. I., Tikkala, A. O. and Mawson, C. A.—(1955) *Canad. J. Biochem. Physiol.*, **33**, 181.

Fleischhauer, K.—(1957) *Naturwissenschaften*, **44**, 589.

Fredricks, R. E., Tanaka, K. R. and Valentine, W. N.—(1960) *J. clin. Invest.*, **39**, 1651.

Gilbert, I. G. F. and Taylor, D. M.—(1956) *Biochim. biophys. Acta*, **21**, 545.

Graig, F. A. and Siegel, E.—(1960) *Proc. Soc. exp. Biol., N.Y.*, **104**, 391.

Gunn, S. A. and Gould, T. C.—(1956) *Ibid.*, **92**, 17.—(1957) *Anat. Rec.*, **128**, 41.

Iidem, Ginori, S. S. and Morse, J. G.—(1955) *Proc. Soc. exp. Biol., N.Y.*, **88**, 556.

Heath, J. C.—(1949) *Nature*, **164**, 1055.

Idem and Liquier-Milward, J.—(1950) *Biochim. biophys. Acta*, **5**, 404.

Herring, W. B., Leavell, B. S., Paixao, L. M. and Yoe, J. H.—(1960) *Amer. J. clin. Nutr.*, **8**, 855.

Hoare, R., Delory, G. E. and Penner, D. W.—(1956) *Cancer*, **9**, 721.

KERR, W. K., KERESTECI, A. C. AND MAYOH, H.—(1960) *Ibid.*, **13**, 550.
McCANCE, R. A. AND WIDDOWSON, E. W.—(1942) *Biochem. J.*, **36**, 392.
McISAAC, R. J.—(1955) *Endocrinology*, **57**, 571.
MAGER, M., McNARY, W. F., Jr. AND LIONETTI, F.—(1953) *J. Histochem. Cytochem.*, **1**, 493.
MAWSON, C. A. AND FISCHER, M. I.—(1952) *Canad. J. med. Sci.*, **30**, 336.
MESCHAN, I., QUINN, J. L., WITCOFSKI, R. L. AND HOSICK, T. A.—(1959) *Radiology*, **73**, 62.
MILLAR, M. J., VINCENT, N. R. AND MAWSON, C. A.—(1961) *J. Histochem. Cytochem.*, **9**, 111.
NATIONAL BUREAU OF STANDARDS.—(1959) Handbook 69. 'Maximum Permissible Body Burdens and Maximum Permissible Concentrations of Radionuclides in Air and in Water for Occupational Exposure'. Washington (U.S. Government Printing Office).
PELC, S. R.—(1956) *Int. J. appl. Radioisotopes*, **1**, 172.
PROUT, G., Jr., SIERP, M. AND WHITMORE, W. F., Jr.—(1959) *J. Amer. med. Ass.*, **169**, 1703.
RIXON, R. H. AND WHITFIELD, J. F.—(1959) *J. Histochem. Cytochem.*, **7**, 262.
ROSENFELD, I. AND TOBIAS, C. A.—(1951) *J. biol. Chem.*, **191**, 339.
ROSS, J. F., EBAUGH, F. G., Jr. AND TALBOT, T. R., Jr.—(1958) *Trans. Ass. Amer. Physcns.*, **71**, 322.
SHELINE, G. E., CHAIKOFF, I. L., JONES, H. B. AND MONTGOMERY, M. L.—(1943) *J. biol. Chem.*, **149**, 139.
SIMMONDS, M.—(1918) *Frankfurt. Z. Path.*, **21**, 178.
THIERS, R. E. AND VALLEE, B. L.—(1957) *J. biol. Chem.*, **226**, 911.
TIPTON, I. H.—(1960) The Distribution of Trace Metals in the Human Body. *In* 'Metal-Binding in Medicine'. Ed. Seven, M. J. and Johnson, L. A. Philadelphia (Lippincott).
VALLEE, B. L.—(1955) Zinc and Metalloenzymes. *In* 'Advances in Protein Chemistry'. Ed. Anson, M. L., Bailey, V. and Edsall, J. T. New York (Academic Press).—(1959) *Physiol. Rev.*, **39**, 443.
Idem AND GIBSON, J. G.—(1948) *J. biol. Chem.*, **176**, 445.
VIKBLADH, I.—(1951) *Scand. J. clin. lab. Invest.*, Suppl., **2**, 9.
WETTERDAL, B.—(1958) *Acta Radiol.*, Suppl., **156**, 1.
WIDDOWSON, E. W., McCANCE, R. A. AND SPRAY, C. M.—(1951) *Clin. Sci.*, **10**, 113.

THE SIGNIFICANCE OF THE BLOOD CONTENT OF THE EHRLICH ASCITES CARCINOMA

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It has been shown previously (Hartveit, 1961) that the adult mice used at this Institute show either a short survival time and a very haemorrhagic tumour or a long survival time and a relatively asanguinous tumour, following the intraperitoneal injection of Ehrlich's ascites carcinoma. This negative correlation between the survival time and the blood content of the tumour is not dependent on the sex or weight of the mouse. As this tumour is said to grow progressively in almost all strains of mice (Karnofsky, 1953) it was decided to investigate the cause of this difference in survival time and blood content found in our mice.

In 1934 Apitz tested the theory that haemorrhage into the solid Ehrlich carcinoma might be due to anaphylaxis, but he failed to produce haemorrhage by this means. He also failed to produce haemorrhage as the result of treatment with histamine. Later Barrett (1942) investigated the relationship between anaphylaxis and sarcoma 37. He was able to show that the anaphylactic shock produced in previously sensitized mice in response to horse serum was accompanied by haemorrhage into actively growing transplants of this tumour. The reaction did not occur in non-sensitized animals. He also produced haemorrhage into the tumour in strain A mice in response to intraperitoneal histamine. The haemorrhagic reaction was not confined to the tumour but was also present, to a lesser extent, in the stomach and small intestine. Barrett's experiment suggests that haemorrhage into a tumour may be the result of the combination of a foreign antigen with antibody present in the tumour.

The mice used in this Institute are heterozygous and also of different genetic make-up from Ehrlich's ascites carcinoma. It was thought that their differences in reaction to the tumour might represent different degrees of genetic difference, and thus different degrees of resistance to the homotransplant.

Ludford (1931) and Andervont (1936) have shown that resistance to tumour transplants can be abrogated by vital staining with trypan blue. More recently many authors (reviewed by Toolan, 1953) have shown that it is possible for homotransplants to grow in animals treated with cortisone. The following experiment was planned in an attempt to reduce the resistance of mice to a tumour homotransplant (i.e. intraperitoneal Ehrlich's ascites carcinoma) by both the methods mentioned above, to see if this treatment would influence the survival time of the mice and the blood content of the tumour ascites.

MATERIAL AND METHODS

The mice used were taken from a closed colony of previously inbred white mice. All were adult, under 6 months old. The Ehrlich ascites carcinoma was originally

obtained from Professor Ahlström in Lund, and, at the time of the experiment, was in its 70th transplant generation here.

Three groups of mice (I, II and III), each containing 15 males and 15 females, were numbered 1–15 for each sex in each group; mice of the same sex and weight (to the nearest gram) in each group receiving the same number. In this way the mean starting weight of the animals in all 3 groups was the same, being 20·8 g. (S.D. 1·237).

Each of the mice was given one intraperitoneal injection of 0·1 ml. of Ehrlich's ascites carcinoma taken from a male mouse of the 70th transplant generation. This mouse, which had received the tumour 10 days before, had 9·5 ml. of tumour ascites which was slightly blood stained, with a tumour cell count of 2,050,000/cu. mm. The cells all appeared viable, i.e. they did not take up eosin from a 1 : 2,000 solution of eosin in Tyrode's solution (Schrek, 1936); they did not clump and showed no abnormalities in films stained with haematoxalin and eosin. When the injections were complete, about one hour after the donor mouse had been killed, the tumour cells in the remaining fluid were all still viable.

Some of the mice were also given subcutaneous treatment as follows:—

Group I—the control group—received no subcutaneous treatment.

Group II—the vitally stained group—were each given 0·5 ml. of a 0·5 per cent solution of trypan blue in sterile distilled water, subcutaneously on the back. The first injection of trypan blue was given 7 days before the mice were injected with tumour ascites. The trypan blue injections were repeated 2 and 4 days later, and thereafter weekly until all the mice in the group were dead.

Group III—the cortisone treated group—received a suspension of cortisone acetate as a subcutaneous injection on the back. The dose was equivalent to 25 mg./kg. starting weight. The first injection was given 6 days before the mice were injected with tumour ascites. The cortisone injections were repeated daily until all the mice in the group were dead.

Three additional control groups were set up (1, 2 and 3). Each of these groups consisted of 5 male and 5 female mice of similar age and weight to those in the three main groups. Each of these groups was given the same subcutaneous treatment as the corresponding Roman figure group (i.e. none, trypan blue, and cortisone, respectively), but none of the mice were injected with tumour ascites.

The survival time of each mouse that died in every group was recorded. When the mice in the Roman figure groups died the tumour ascites was removed and 1 ml. of this centrifuged at 2,400 r.p.m. for 45 minutes. The volume of the red blood cells at the bottom of the Wintrobe tube was taken as an estimate of the amount of blood present/ml. and recorded as a percentage of the total volume in the tube.*

RESULTS

Table I shows the sex difference in the survival time and blood content of the tumour ascites in the mice in groups I, II and III, all of which died. The table gives the values for the total, male and female series, with the number of mice, the mean value with S.D., the S.E. of the actual difference between the male and female means, and the t and P values for this difference.

* One mouse in group II died of trauma the day after the injection of the tumour and was excluded from subsequent calculations.

TABLE I.—*The Sex Differences in Survival Time and Blood Content of the Tumour Ascites in the Three Groups*

x	Group	Series	n	$\bar{x}$	S.D.$\bar{x}$	S.E. difference $\bar{x}_\delta$ & $\bar{x}_\varphi$	t	P
Survival time (days)	I	♂+♀	30	11·03	2·683			
		♂	15	10·8	2·186	1·013	0·4862	0·7>P>0·6
		♀	15	11·3	3·253			
	II	♂+♀	29	12·24	3·332			
		♂	14	12·9	2·901	1·33	0·9773	0·4>P>0·3
		♀	15	11·6	4·188			
	III	♂+♀	30	14·93	4·528			
		♂	15	11·8	3·571	1·233	5·077	0·001>P
		♀	15	18·06	3·17			
Blood content of ascites (vol. %)	I	♂+♀	30	4·3	4·638			
		♂	15	4·4	5·144	1·736	0·0806	P>0·9
		♀	15	4·26	4·331			
	II	♂+♀	29	1·717	4·286			
		♂	14	2·036	3·782	1·702	0·2974	0·8>P>0·7
		♀	15	1·53	5·304			
	III	♂+♀	30	1·216	2·616			
		♂	15	0·9	2·569	0·9806	0·6424	0·6>P>0·5
		♀	15	1·53	2·798			

The number of mice in each group and series is shown, the mean value for the factor under consideration (x) with its S.D., the S.E. of the actual difference between male and female means, and the t and P values for this difference.

The mean survival time for the total series was shortest in the control group I (11·03 days, S.D. 2·683), and longest in the cortisone treated group III (14·93 days, S.D. 4·528). The vitally stained group II fell between these two extremes (12·24 days, S.D. 3·332). The sex difference within the groups was not statistically significant in groups I and II (0·5 and 1·3 days respectively), while it was highly significant in group III (6·26 days, 0·001 > P). The mean blood content of the tumour ascites was highest in the control group I (4·3 per cent, S.D. 4·638), and lowest in the cortisone treated group III (1·216 per cent, S.D. 2·616). Once again the vitally stained group II showed an intermediate value (1·717 per cent, S.D. 4·286). The sex differences were not significant.

TABLE II*a*.—*Comparison of the Differences in Survival Time Between the Three Groups*

Comparison between groups x and y		Series	n_x	n_y	Difference between $\bar{x}$ and $\bar{y}$	S.E. difference between $\bar{x}$ and $\bar{y}$	t	P
Survival time (days) I	II	♂+♀	30	29	1·21	0·7891	0·1533	0·9>P>0·8
I	III	♂	15	15	1·0	4·187	0·2388	0·9>P>0·8
I	III	♀	15	15	6·76	1·172	5·768	0·001>P
II	III	♂	14	15	1·1	1·204	0·9137	0·4>P>0·3
II	III	♀	15	15	6·46	1·356	4·764	0·001>P

The difference in survival time between the groups for the total series, or for the male and female series separately when their difference is significant, is shown, with the number of mice used and the S.E., t and P values for the difference.

Table II*a* compares the differences in survival time between the three main groups, giving the difference between the groups for the total series, or for the male

and female series separately when their difference is significant, with the number of mice used, the S.E., t and P values for the difference.

The difference in survival time of the mice in groups I and II, total series (1·21 days) is not statistically significant In groups I and III this difference (1 day) is not significant for the male mice, but the difference for the female mice in these groups (6·67 days) is highly significant ($0{\cdot}001 > P$). The difference between groups II and III for the male mice (1·1 days) is not significant, but that for the females (6·46 days) is highly so ($0{\cdot}001 > P$).

TABLE II*b*.—*Comparison of the Differences in Blood Content of the Tumour Ascites Between the Three Groups*

	Comparison between groups x and y		Series	n_x	n_y	Difference between $\bar{x}$ and $\bar{y}$	S.E. difference between $\bar{x}$ and $\bar{y}$	t	P
Blood content of ascites (vol. %)	I	II	♂+♀	30	29	2·583	1·161	2·224	$0{\cdot}05 > P > 0{\cdot}02$
	I	III	♂+♀	30	30	3·184	0·9705	3·281	$0{\cdot}01 > P > 0{\cdot}001$
	II	III	♂+♀	29	30	0·501	0·7416	0·675	$0{\cdot}5 > P > 0{\cdot}4$

The difference in blood content between the groups is shown for the total series, with the number of mice used, the S.E., t and P values for the difference.

Table II*b* compares the differences in blood content of the tumour ascites between the three main groups for the total series, giving the number of mice used, the S.E., t and P values for the difference. The difference between groups I and II (2·583 per cent) is significant ($0{\cdot}05 > P > 0{\cdot}02$), while that between groups I and III (3·184 per cent) is highly so ($0{\cdot}01 > P > 0{\cdot}001$). The difference between groups II and III (0·7416 per cent) is not significant.

The relationship between the survival time and the amount of blood in the tumour ascites in the three main groups is shown in Table III, which gives the

TABLE III.—*The Relationship Between the Survival Time and the Blood Content of the Tumour Ascites in the Three Groups*

Group	Series	n	r	S.E.r	t	P
I	♂+♀	30	−0·8381	0·2397	3·496	$0{\cdot}01 > P > 0{\cdot}001$
	♂	15	−0·9831	0·2673	3·678	
	♀	15	−0·8708	0·2673	3·258	
II	♂+♀	29	−0·5125	0·1889	2·713	$0{\cdot}02 > P > 0{\cdot}01$
	♂	14	−0·4658	0·2773	1·680	$0{\cdot}2 > P > 0{\cdot}1$
	♀	15	−0·7591	0·2673	2·840	$0{\cdot}02 > P > 0{\cdot}01$
III	♂+♀	30	−0·01574	0·2397	0·06567	$P > 0{\cdot}9$
	♂	15	−0·02207	0·2673	0·08258	
	♀	15	−0·2633	0·2673	0·9851	$0{\cdot}4 > P > 0{\cdot}3$

The number of mice in each group and series is shown, and the correlation coefficient (r) with its S.E., t and P values.

number of mice in each group and series, the correlation coefficient (r), with its S.E., t and P values. In the control group I the amount of blood in the tumour ascites decreased with survival time. This negative correlation (−0·8381, −0·9831, −0·8708 for the total, male and female series, respectively) was highly significant in all cases ($0{\cdot}01 > P > 0{\cdot}001$). These findings are in keeping with those in the

author's previous experiment (Hartveit, 1961). In the vitally stained group II the negative correlation was greatly reduced (−0·5125, −0·4658, −0·7591, respectively), being significant for the total and female series ($0{\cdot}02 > P > 0{\cdot}01$). It was even further reduced in the cortisone treated group III (−0·01574, −0·02207, −0·2633) and as such was not significant.

With regard to the control groups 1, 2 and 3 ; none of the mice in group 1 died. One of the female mice in group 2 died 12 days after its first injection of trypan blue. One male and one female mouse in group 3 died after 21 injections of cortisone.

DISCUSSION

In this experiment there are three factors to be considered : the tumour, the relationship of the mice to the tumour and the interrelationship of the mice themselves.

As regards the tumour, Klein and Révész (1953) have shown that provided a sufficient tumour cell dose is used Ehrlich's ascites carcinoma can be expected to grow progressively, and the mouse to die within a time limit dependent on that tumour cell dose. The tumour cell dose used in the present experiment is comparable to that used in the author's previous experiment (Hartveit, 1961). The adult mice were taken from the same closed colony. On comparing the mice in group I of the present experiment to those in the previous experiment it is found that the actual difference in their mean survival time (0·53 days) is not significant ($0{\cdot}5 > P > 0{\cdot}4$). The difference in the mean blood content (1·7 per cent) is not significant either ($0{\cdot}2 > P > 0{\cdot}1$). In both cases the negative correlation between the survival time and the blood content of the tumour ascites is highly significant. Thus, although the tumour came from different transplant generations, there was no significant difference in the survival time or blood content, and the relationship between these two factors was unchanged. These facts support the view that the tumour can, in this case, be regarded as a relatively constant factor. In the present experiment all the tumour used came from one mouse, and this is an additional safeguard to the assumption that the tumour is a constant.

The mice were, of necessity, of different genetic make-up to the tumour, as Ehrlich's ascites carcinoma came originally from a heterozygous mouse (Snell, 1953). Therefore, one would expect that the mice would not accept transplants. But Ehrlich's ascites carcinoma is one of the so-called " non-specific " tumours that is said to contain fewer antigens than usual and so is less demanding than most in its choice of host (Barrett, 1958). However, a perfect fit between host and tumour cannot be expected. While the fit may be close enough to allow the tumour to grow the difference may show up in other ways—for example in the stromal reaction.

Thus, although the mice were not, as in Barrett's experiment referred to earlier (Barrett, 1942), sensitized beforehand with a foreign antigen, the tumour may possess tissue antigens that the mice lack. If they then react against this foreign protein their immunological response might be expected, on the basis of Barrett's findings, to be accompanied by haemorrhage into the tumour, i.e. by a change in the stromal reaction.

This hypothesis is based on the assumption that resistance to homotransplants is genetically determined. This has been shown to be true in the case of red blood cells (Cushing and Campbell, 1957) and normal tissues (Loeb and Wright, 1927 ;

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Billingham, Brent, Medawar and Sparrow, 1954). The Mendelian nature of the genetic influences determining susceptibility to transplanted tumours has also been established (Little, 1956).

When a tumour is transplanted the tumour cells reproduce, the stroma is supplied by the host (Muir, 1951). In the case of Ehrlich's ascites carcinoma the stroma is represented by the ascitic fluid, which contains a variable number of white blood cells. In addition, in some cases, this stroma contains large numbers of red blood cells (Loewenthal and Jahn, 1932 : Kun, Talalay and Williams-Ashman, 1951).

These red blood cells have appeared in the stroma either in direct response to the tumour cells, or in response to the mouse's reaction to these cells. If the first is the case we would expect that the amount of blood in the stroma would be constant for a given tumour cell dose. But the author has previously shown (Hartveit, 1961) that the amount of blood varies inversely with the survival time in mice receiving the same tumour cell dose. If the second is then true, the amount of blood should vary in accordance with the genetic dissimilarity between the mouse and the tumour.

Genetic dissimilarity is evidenced as resistance to transplantation (Snell, 1957). It has been shown that this resistance can be abrogated (Ludford, 1931 ; Andervont, 1936). The mice in the present experiment were treated in such a way as to abrogate their resistance to the transplantation of foreign tissue.

On the basis of the above reasoning one would expect that by supressing host resistance one would change the amount of blood present in the stroma : an increase indicating that the blood was an expression of the mouse's lack of resistance to the tumour, a decrease that it was an expression of its resistance. On the basis of Barrett's experiment (Barrett, 1942), one would expect a decrease.

The mice used were heterozygous. This does not affect their individual relationship to the tumour as discussed above, but different degrees of reaction are to be expected—those nearer to the tumour gentically reacting less against it. As the mice were from the same closed colony it is reasonable to assume that the genetic range in the three samples should be similar, and the results following abrogation of their natural resistance comparable.

All the mice receiving Ehrlich's ascites carcinoma in the present experiment were given the same dose of viable tumour cells. The dose used was chosen as it was known to give an ascitic tumour and a relatively short survival time. The dosage of trypan blue was based on that used by Andervont (1936), and the cortisone dosage on that used by Hobson (1960).

The results in the control mice show that the subcutaneous treatment alone will not influence the survival times of the experimental groups to any appreciable extent. Only one of the mice receiving trypan blue died during the course of the experiment and two of those given cortisone died towards its end.

The results in groups I, II and III show that, although all the mice died, their survival times and the blood content of their tumours differed considerably. Trypan blue did not alter the survival time of the mice but it did reduce the amount of blood in the tumour ascites. Cortisone increased the survival time of the female mice, and greatly reduced the amount of blood in the ascites in both sexes. In addition trypan blue, in part, and cortisone completely, abolished the negative correlation between the survival time and the amount of blood in the tumour ascites that was so strongly apparent in the control group.

Thus, by using methods claimed to reduce the natural resistance of the mice to tumour transplantation we have succeeded in increasing their survival time and decreasing their stromal reaction to the tumour. The blood content of the tumour must, therefore, be considered as an expression of resistance to Ehrlich's ascites carcinoma. We are then left with the paradoxical situation in which the mice with the greatest and most rapid local reaction to the tumour—indicating a type of resistance—die before those without such resistance. Those dying early died as a result of their immunological reaction to the homotransplant, while those surviving longer died as a result of their acceptance of the tumour that was able to grow progressively until it killed the host mechanically.

This finding that the blood content of the tumour is an expression of the resistance of the mouse to the tumour may be of practical use in the field of tumour immunity. In 1958 Barrett stated that antibodies " have eluded detection " in this field. Since then *in vitro* tests for haemagglutinins (Feldman and Sachs, 1957), the tanned erythrocyte technique (Finney, Byers and Wilson, 1960), complement fixation (Lund, 1958) and skin tests (Grace and Dao, 1958) have been reported to give positive results. However, most evidence of such immunity is circumstantial. Thus when an animal is immunized against a tumour the test of whether immunity has been produced is whether or not a transplant of that tumour will be able to grow in the host. Other methods involve the use of an immune serum from a foreign host (Flax, 1956). In this case the antibody is an antibody to a foreign protein, and is not dependent on the fact that the cells were tumour cells. With Ehrlich's ascites carcinoma the amount of blood in the ascites may prove to be a yard-stick by which it will be possible to measure induced as well as natural immunity to the tumour. Experiments in this direction are in progress.

It may be that this haemorrhagic reaction is a Shwartzman-like phenomenon. It has been suggested that the Shwartzman phenomenon may be a manifestation of an immune response (Thomas, 1954). Stetson (1955) has shown that injection of bacterial endotoxin can elicit haemorrhage in skin areas previously prepared by the intradermal injection of homologous or heterologous bacterial products, and compares the reaction to that following the injection of tuberculin in specifically sensitized rabbits. He postulates that both reactions involve a delayed type of allergy, that is accompanied by focal (haemorrhagic) reactions, and systemic reactions—following intraperitoneal injection of the endotoxin—that may be fatal. Lawrence (1956) has drawn analogies between this type of delayed sensitivity and homograft rejection. In the present experiment it has been shown that the haemorrhagic response occurring in some of the mice following transplantation of the tumour is dependent on their natural resistance to the transplant. This response can be likened to the Shwartzman phenomenon in that the mice were previously sensitive to the transplant and showed focal haemorrhage, and a severe systemic reaction leading to death, following a massive intraperitoneal dose of the incompatible material.

SUMMARY

Three groups of 30 heterozygous mice were injected with Ehrlich's ascites carcinoma. Two of the groups of animals were treated with trypan blue and cortisone, respectively, in an attempt to abrogate their natural resistance to the tumour. The amount of blood in the tumour ascites was found to be less following these treatments. (Average per cent : Control—4·3, trypan blue—1·7, cortisone—1·2).

Thus the blood content of the tumour ascites can be regarded as an expression of the animal's reaction against, i.e. natural resistance to, the tumour. It was also shown that the survival time of the mice that did not react was greater than that of those which reacted against the tumour—suggesting that the latter died as a result of their immunological reaction to an overwhelming dose of foreign tissue. It is possible that this reaction is comparable to a Shwartzman-like phenomenon.

REFERENCES

ANDERVONT, H. B.—(1936) *Publ. Hlth Rep., Wash.*, **51**, 591.

APITZ, K.—(1934) *Z. Krebsforsch.*, **40**, 50.

BARRETT, M. K.—(1942) *J. nat. Cancer Inst.*, **2**, 625.—(1958) *J. chron. Dis. (St. Louis)*, **8**, 136.

BILLINGHAM, R. E., BRENT, L., MEDAWAR, P. B. AND SPARROW, E. M.—(1954) *Proc. roy. Soc.* B, **143**, 43.

CUSHING, J. E. AND CAMPBELL, D. H.—(1957) 'Principles of Immunology'. London (McGraw Book Company, Inc.).

FELDMAN, M. AND SACHS, L.—(1957) *J. nat. Cancer Inst.*, **18**, 529.

FINNEY, J. W., BYERS, E. G. AND WILSON, R. H.—(1960) *Cancer Res.*, **20**, 351.

FLAX, M. H.—(1956) *Ibid.*, **16**, 774.

GRACE, J. T. AND DAO, T. L.—(1958) *Surg. Forum*, **9**, 611.

HARTVEIT, F.—(1961) *Brit. J. Cancer*, **15**, 336.

HOBSON, D.—(1960) *Brit. J. exp. Path.*, **41**, 251.

KARNOFSKY, D. A.—(1953) 'Experimental Cancer Chemotherapy'. In 'The Physiopathology of Cancer', ed. Homburger, F. and Fishman, W. H. New York (Paul B. Hoeber, Inc.), p. 635.

KLEIN, G. AND RÉVÉSZ, L.—(1953) *J. nat. Cancer Inst.*, **14**, 229.

KUN, E., TALALAY, P. AND WILLIAMS-ASHMAN, H. G.—(1951) *Cancer Res.*, **11**, 855.

LAWRENCE, H. S.—(1956) *Amer. J. Med.*, **20**, 428.

LITTLE, C. C.—(1956) 'The Genetics of Tumor Transplantation'. In 'Biology of the Laboratory Mouse', ed. Snell, G. D. London (Constable and Co. Ltd.), p. 279.

LOEB, L. AND WRIGHT, S.—(1927) *Amer. J. Path.*, **3**, 251.

LOEWENTHAL, H. AND JAHN, G.—(1932) *Z. Krebsforsch.*, **37**, 437.

LUDFORD, R. J.—(1931) *Brit. J. exp. Path.*, **12**, 108.

LUND, H. J. C.—(1958) *Acta path. microbiol. scand.*, **43**, 391.

MUIR, R.—(1951) 'Textbook of Pathology'. 6th ed., revised Cappel, D. F. London (Arnold and Co.), p. 280.

SCHREK, R.—(1936) *Amer. J. Cancer*, **28**, 389.

SNELL, G. D.—(1953) 'Transplantable Tumors'. In 'The Physiopathology of Cancer', ed. Homburger, F. and Fishman, W. H. New York (Paul B. Hoeber, Inc.), p. 343.

SNELL, G. D.—(1957) *Ann. N.Y. Acad. Sci.*, **69**, 555.

STETSON, C. A., Jr.—(1955) *J. exp. Med.*, **101**, 421.

THOMAS, L.—(1954) *Ann. Rev. Physiol.*, **16**, 467.

TOOLAN, H. W.—(1953) *J. nat. Cancer Inst.*, **14**, 745.

SULPHYDRYL GROUPS AND TUMOUR INDUCTION BY CHEMICAL AGENTS

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AMONG other specific fields the role of sulphydryl groups in both tumorigenesis and the developed tumour has received considerable attention. Because of technical difficulties associated with the measurement of –SH groups within tissues much of this work has only been via indirect approaches. Recently Calcutt and Doxey (1959) described a technique which gives quantitative measurements of free –SH groups within tissues. Further application of this technique during studies with carcinogenic and related agents has led to the hypothesis that an elevation in the target tissue sulphydryl level is an essential feature of tumour induction—Calcutt, Doxey and Coates (1961*a*).

The purpose of the present paper is to summarise the evidence for this hypothesis and to examine the hypothesis in the light of other evidence available in the literature. It must be stressed that all data and conclusions referred to are in association with the induction phase of tumours and are not applicable to the formed tumour.

Evidence for a rise in tissue –SH levels during tumour induction

Measurements of sulphydryl levels fall into three main classes, namely :—
measurements of reduced glutathione :
measurements of free tissue –SH plus reduced glutathione :
measurements of free tissue –SH.

Additionally there are in the literature a number of examples of measurements after tissue denaturation, as by homogenisation, grinding procedures, or fixation. These have been disregarded as not being any approximation to the state of the normal tissue.

Findings relative to total –SH or glutathione –SH in susceptible tissues treated with chemical carcinogens in a manner likely to induce tumour formation are summarised in Table I. In all cases elevations in –SH levels have been recorded and usually over periods ranging from several weeks to several months. In limited series of measurements on rat livers treated with N.2-fluoroenylacetamide or p-dimethyl-aminoazobenzene, Calcutt, Doxey and Coates (1960) found that rises in total –SH levels were comprised of rises in both protein bound and glutathione –SH.

A number of other records of –SH measurements during tumour induction also occur in the literature. Crabtree (1946) failed to find any alterations in the glutathione content of mouse skin after painting with 3,4-benzopyrene or

TABLE I.—*Effects of Carcinogens on Target Tissue –SH Levels*

Carcinogen	Animal	Tissue	Treatment	Effect on —SH	Period of effect	Reference
3,4-Benzopyrene	Mouse	Skin	Once weekly 0·5% soln.	Total up	9–50 days	*a*
1,2 : 5,6-Dibenzanthracene	,,	,,	Twice weekly 0·1% soln.	,, ,,	8–78 ,,	*b*
7,12-Dimethylbenzanthracene	,,	,,	Ditto	,, ,,	8–36 ,,	*b*
1,2 : 5,6-Dibenzacridine	,,	,,	Once weekly 0·3% soln.	,, ,,	1–120 ,,	*c*
N.2 Fluorenylacetamide	Rat	Liver	0·4% in diet	,, ,,	6–90 ,,	*a*
		Kidney	,, ,, ,,	Slight rise	20–41 ,,	*a*
p-Dimethylaminoazobenzene	,,	Liver	0·6% in diet	Total up	27–48 ,,	*a*
p-Dimethylaminoazobenzene	,,	,,	?	GSH up	Precancerous period	*d*
p-Dimethylaminoazobenzene	,,	,,	?	,, ,,	In early experimental period	*e*
3′-Methyl, 4-dimethylaminoazobenzene	,,	,,	0·6% in diet	,, ,,	30–80 days	*f*
Urethane	Mouse	Lung	0·1% in water	Total up	6–69 ,,	*a*
o-Aminoazotoluene	,,	Liver	8 mgm. subcut.	,, ,,	2–72 ,,	*c*
o-Aminoazotoluene	,,	Lung	,, ,,	,, ,,	1–65 ,,	*c*
Carbon tetrachloride	,,	Liver	0·005 ml. twice weekly	,, ,,	23–72 ,,	*c*
Choline deficiency	Rat	,,	Dietary deletion of weight	,, ,,	2–30 ,,	*g*
3,4 : 5,6-Dibenzocarbazole	Mouse		12·5 mg./Kg.	GSH up	4–180 ,,	*h*
Ethionine	Rat	,,	0·2% in diet	Total up	2–79 ,,	*i*

In the above table—total refers to free tissue —SH plus reduced glutathione and GSH to reduced glutathione.

References.—*a*, Calcutt, Doxey and Coates (1960); *b*, Calcutt and Coates (1961); *c*, Calcutt, Doxey and Coates (1961*a*); *d*, Masayama, Iki, Yokayama and Hasimoto (1938); *e*, Iki (1939); *f*, Fiala and Fiala (1959); *g*, Byerrum, Erway and Dubois (1948); *h*, Boyland and Mawson (1938); *i*, Calcutt (1961*b*).

1,2 : 5,6-dibenzanthracene, but these experiments only extended over a period of 4 hours. With measurements extending over periods of up to five days, Di Paolo and Niedbala (1957) found elevated mouse skin –SH levels after treatment with 1,2 : 5,6-dibenzanthracene or 7,12-dimethylbenzanthracene. Schultze (1960) gave 1·0 ml. of carbon tetrachloride subcutaneously to male rats and nine days later the livers showed a " significant –SH increase." In an experiment in which kidney total –SH was measured on hamsters after stilboestrol implants Calcutt *et al.* (1960) found high –SH values (at 39 and 74 days after commencement of experiment) in two animals in which the implant had been almost totally absorbed. The remaining animals showed no sign of absorption of the implant and –SH levels were slightly below the control values. Gallico and Boretti (1948) made –SH measurements on the livers of rats receiving *o*-aminoazotoluene. Estimations were made at intervals of several months and no allowance was made for the effects of a restricted diet (rice), so no conclusion can be drawn from these figures.

The evidence so far is weighty but does not exclude the possibility that the effect is a purely non specific one. In this event it might be anticipated that chemical carcinogens would display a similar response in tissues which are not susceptible to the carcinogenic activity of the agent in question. Findings in this respect have been summarized in Table II. Despite two cases in which slight

TABLE II.—*Effects of Carcinogens on Non-target Tissue –SH Levels*

Carcinogen	Animal	Tissue	Effect on –SH	Period of observation	References
3,4-Benzopyrene	Mouse	Liver	No change	65 days	*a*
3,4-Benzopyrene	,,	Kidney	,, ,,	65 ,,	*a*
3,4-Benzopyrene	Rat	Liver	Slight decrease	30 ,,	*j*
1,2 : 5,6-Dibenzanthracene	Mouse	,,	No change in GSH	10 ,,	*b*
20-Methylcholanthrene	,,	,,	Ditto	20 ,,	*b*
N.2 Fluorenylacetamide	Rat	Cardiac muscle	Slight increase	104 ,,	*a*
p-Dimethylaminoazobenzene	,,	Kidney	No change	76 ,,	*a*
Urethane	Mouse	Skin	Decrease	69 ,,	*a*
Stilboestrol	Hamster	Liver	,,	87 ,,	*a*
o-Aminoazotoluene	Mouse	Kidney	No change	72 ,,	*c*
Carbon tetrachloride	,,	,,	Slight rise	72 ,,	*c*

References as in Table I and additionally, *j*—Rondoni and Boretti (1947).

increases in sulphydryl levels have been recorded the overall impression must be that, so far as information is available, chemical carcinogens have little effect on the –SH level of non-target tissues.

In short term experiments (up to 6 hours) Wood and Kraynak (1953) found a fall in the plasma –SH level of dogs which had been injected with 3,4-benzopyrene. This may be merely a reflection of the fall in liver –SH levels which Calcutt, Doxey and Coates (1959) found to occur temporarily after intravenous injection of 3,4-benzopyrene into mice.

Another possibility which must be considered is that agents chemically related to carcinogens would have similar effects to the carcinogens in susceptible tissues. Here the evidence is very limited. In the case of mouse skin Calcutt and Coates (1961) have found that repeated paintings with anthracene or pyrene will induce slight falls in –SH levels or have no effect.

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The evidence so far, although not conclusive, does favour the view of a rise in tissue –SH levels in susceptible tissues being associated with carcinogenesis. It is now necessary to examine some other factors involved in this problem.

Some miscellaneous factors affecting tissue –SH levels

The distribution and availability of sulphydryl groups within tissues is liable to be influenced by the physiologic state of the animal or tissue in question. It has been found by Calcutt, Doxey and Coates (1961*b*) that deletion of riboflavin from the diet of mice causes a rise in liver –SH levels, whilst the injection of riboflavin causes a rapid and sustained fall in –SH levels. Deletion of pantothenate or thiamine from the diet also causes temporary rises in skin –SH levels (Calcutt, 1961*a*). A rise in the glutathione content of rabbit muscles can be brought about by the injection of pituitary growth hormone (Gregory and Goss, 1934 ; Goss and Gregory, 1935). In a more recent review, Lazarow (1954) summarises an extensive literature and shows that hypophysectomy causes a lowering of glutathione levels in most tissues. Glutathione levels in blood and tissues are also influenced by adrenal, thyroid, pancreatic and parathyroid hormones (Lazarow, 1954).

An entirely different class of compounds has also been found, capable of influencing tissue –SH levels. These are the cocarcinogenic agents. Calcutt and Coates (1961) have shown that croton oil causes an elevation in mouse skin total free –SH values for up to five weeks during repeated painting. Oleic acid also causes an elevation of mouse skin –SH levels (Calcutt, 1961*c*). An indication that Tween 60 (polyoxyethylene sorbitan monostearate) which is also cocarcinogenic behaves in a similar fashion is given by the finding of Setälä, Äyräpää, Niskanen, Stjernvall and Nyholm (1960) that mouse skin treated with this agent stains more intensively for –SH groups than does normal skin.

Some factors enhancing tumour formation and their relation to sulphydryl groups

During the course of experiments devoted to the experimental production of tumours a number of factors enhancing tumour formation have been elicited.

Tannenbaum (see review by Tannenbaum and Silverstone (1957)), has concluded that overfeeding increases the yield of experimental tumours. The major action was found to occur during the developmental phase, i.e. the period during which rises in –SH levels occur (Table I). If the rise in –SH involved extra cellular requirements for sulphur containing compounds then this could be provided by the additional feeding of the animal during this period. Baumann (1948) has pointed out that dietary supplements of cystine augment the formation of a variety of tumours. This again could be the supplying of a basic substrate required for tissues to develop a high –SH level.

Another factor which enhances tumour development is a diet high in certain fats. This will influence tumour formation after treatment with *p*-dimethylaminoazobenzene (Kline, Miller, Rusch and Baumann, 1946), or polycyclic hydrocarbons (Tannenbaum, 1944). In this latter case it has been shown that the animals on a high fat diet eat more and thus a high calorie intake is achieved leading to a situation similar to that considered in the previous paragraph. Even when allowance is made for this increased calorie intake there is still a slight augmentation. A possible clue to this effect may lie in the finding by

Kaunitz, Johnson and Slanetz (1952) that rancid fat can accelerate the utilisation of riboflavin, an agent known to be protective against certain types of tumour formation and also able to affect –SH levels (see above).

The best known example of an enhancing factor is the use of croton oil in skin painting experiments after a minimal dose of carcinogen. Calcutt and Coates (1961) have shown that after a single application of 7,12-dimethylbenzanthracene to mouse skin there is only a transient rise in the skin –SH level and then a return to the normal level. If this single treatment which would be expected to yield no—or very few—tumours is followed by repeated paintings with croton oil then a sustained rise in –SH levels similar to that achieved by repeated paintings with dimethylbenzanthracene is achieved. Thus, after an initiating treatment two different agents, one a carcinogen and the other a non carcinogen, produce the same biochemical effect and the same biological result.

Anticarcinogens and sulphydryl groups

Considerable attention has been given at various times to the search for agents which will inhibit the formation of experimental tumours. In a number of cases specific agents have been used and a clear cut evaluation of results is possible, but in many cases the number of factors involved is such as to make interpretation difficult if not impossible.

Although never substantiated experimentally the idea that carcinogens became linked to tissue sulphydryl groups has long been current. On this basis Crabtree (1944, 1945, 1946) investigated a series of compounds known to inhibit sulphydryl groups or to form mercapturates. These included bromobenzene, maleic and citraconic anhydrides, anthracene and phenanthrene. When painted on to mouse skin concurrently with carcinogenic polycyclic hydrocarbons they reduced the expected tumour yields. It was argued that these compounds reduced the availability of tissue –SH for binding with the carcinogen. In the light of evidence considered earlier it can now be suggested that they limit the rise in tissue –SH level induced by the carcinogen. Further findings which may be considered similarly are those of Carruthers (1940), Riley and Wallace (1941) and Riley and Pettigrew (1944), that aldehydes inhibit carcinogenesis since Schubert (1936) has shown aldehydes to react with thiol compounds. Mustard gas is another compound which reacts with –SH groups and this was found by Berenblum (1935) to inhibit the induction of tar tumours.

On the alternate policy of offering sulphydryl which would compete with that in the tissue for carcinogens Crabtree (1948) tested a series of monothiols and also BAL (2 : 3 dimercapto-propanol) against tumour induction by polycyclic hydrocarbons. The monothiols were found to be ineffective but the BAL did show slight activity. This was confirmation of the experience of Lusky, Braun and Woodard (1947) who had obtained a noticeable reduction in the yield of benzopyrene induced mouse skin tumours by concurrent treatment with BAL. Earlier, Reimann and Hall (1936) claimed that thiocresol effectively inhibited carcinogenesis by 1,2 : 5,6-dibenzanthracene but Crabtree (1948) pointed out that under the experimental conditions used this conclusion was not warranted. In connection with these experiments a further point derives from the fact that Calcutt (1949) and Garzia and Dansi (1953) found that benzopyrene was itself oxidised in the presence of autoxidising thiols. Under these circumstances the applica-

tion of a thiol to mouse skin already treated with benzopyrene may have resulted in oxidation of both the carcinogen and thiol and a consequent reduction in the effective dose of carcinogen.

A rather different type of anticarcinogen is Vitamin A. This was found by Rosicky and Hatschek (1943) to diminish the yield of mouse skin benzopyrene tumours when painted concurrently with the carcinogen. It has since been claimed by Flesch and Goldstone (1952) that Vitamin A inactivates epidermal –SH groups. Pelc and Fell (1960) have found excess Vitamin A to severely limit the uptake of cystine throughout the epithelium of chick embryo. This might indicate a limitation of substrate available for the formation of –SH groups.

Reference has already been made to the general lowering of tissue glutathione levels brought about by hypophysectomy. This procedure has also been found to profoundly influence the production of experimental tumours. Both delay in the appearance of a tumour and total suppression of tumour formation have been recorded. The findings in these experiments have been summarised in Table III.

TABLE III.—*The Influence of Hypophysectomy upon Experimental Tumour Induction*

Agent	Animal	Site	Result	Reference
3,4-Benzopyrene	Mouse	Skin	Reduced yield	Kortweg and Thomas (1939).
3,4-Benzopyrene	Rats	Subcut. tissue	Delay in appearance	Agate, Antopol, Glaubach, Agate and Graff (1955).
20-Methylcholanthrene . . .	,,	Muscle	Reduced yield	Moon and Simpson (1955).
9,10-Dimethylbenzanthracene . .	,,	,,	,, ,,	Moon, Li and Simpson (1956).
9,10-Dimethylbenzanthracene . .	,,	,,	Delay in appearance	Noble and Walters (1954).
3'-Methyl, 4-dimethylaminoazobenzene	,,	Liver	Reduced yield	Robertson, O'Neal, Griffin and Richardson (1953).
N.2 Fluoroenyldiacetamide . .	,,	,,	No tumours*	O'Neal, Hoffman, Dodge and Griffin (1958).

* High incidence of tumours of Harderian gland origin.

Human preneoplastic conditions in relation to sulphydryl levels

Gradually it has become recognised that certain human disease states are associated with a high incidence of tumour occurrence.

A well recognised condition is that of oral leucoplakia, and this has been found by Abels, Rekers, Martin and Rhoads (1942) to be associated with dietary insufficiencies of Vitamins A and B_2 (riboflavin) and often to respond to improved diet. Evidence has already been given implicating Vitamin A and B levels with the sulphydryl content of tissues. It is under conditions of low vitamin levels that increased –SH levels might be expected, and in this case there is an association of low vitamin levels with a precancerous state.

A deficiency of riboflavin has also been associated with the Patterson-Kelly (Plummer-Vinson) syndrome by Ahlbom (1936). This condition is believed to predispose to carcinoma of the nasopharynx. Vitamin deficiency has also been incriminated in the aetiology of liver cancer amongst the Bantus (Berman, 1951).

Stocks and Karn (1933) concluded that the regular consumption of milk, green vegetables and possibly wholemeal bread had some kind of protective influence against cancer. It may not be without point that these foodstuffs are major sources of riboflavin.

Sulphydryl levels at the end of the latent period

In the earlier part of this paper, evidence was offered that a rise in the target tissue –SH level occurs during the latent period. This rise was often of a duration much shorter than the latent period for the carcinogen and tissue in question. The question as to what happens to –SH levels up to the appearance of the tumour must be considered. The available evidence is limited. Boyland and Mawson (1938) continued their glutathione estimations on the livers of mice treated with 3,4 : 5,6-dibenzcarbazole until bile duct proliferation occurred. After 120 days most values had returned to the normal level. Fiala and Fiala (1959) in their estimations of glutathione in azodye treated rat liver, found that after the rise period (30–80 days) there was a progressive decline up to 210 days after initiation of treatment. Although the experiments were not carried on long enough there are indications in some of the results of Calcutt, Doxey and Coates (1960, 1961) that after the rise period there is a fall to below normal levels.

There are, additionally, some indications that this reduced –SH level persists into the formed tumour. Thus, Fiala and Fiala (1959) found much lower levels of glutathione in both azo dye induced hepatomas and the Novikoff hepatoma as compared with normal rat liver. Kinosita (1938) found primary rat hepatoma to have a lower glutathione content than rat liver. Greenstein and Leuthardt (1945) found the total cysteine content of four different rat tumours to be rather lower than that of normal rat tissues. Doxey (unpublished data) has found azo dye induced rat hepatoma to have both a lowered glutathione and lowered protein –SH content as compared with normal rat liver. Benzopyrene induced epitheliomata of mouse skin also had a low total –SH content as compared with normal mouse skin. Several transplantable mouse tumours were also found to have rather low total –SH values.

Sulphydryl levels in relation to stages of carcinogenesis

It is now generally accepted that the carcinogenic process involves at least two different stages. These are usually referred to as "initiation" and "promotion". The data which has already been collected must also be considered in terms of these stages in the carcinogenic process.

Initiation usually only requires a single treatment with the carcinogenic agent. It was found by Di Paolo and Niedbala (1957) that single applications of 1,2 : 5,6-dibenzanthracene or 7,12-dimethylbenzanthracene caused temporary –SH rises in mouse skin –SH levels. Calcutt and Coates (1961) obtained a similar result with 7,12-dimethylbenzanthracene. Di Paolo and Niedbala (1957) also recorded slight falls in –SH level shortly after application of the hydrocarbons. These may have been associated with metabolism of the hydrocarbons since Calcutt, Doxey and Coates (1959) associated similar slight falls in mouse liver –SH values after treatment with carcinogenic hydrocarbons with detoxication processes. Examination of the figures in Table I suggests that in many cases there has been no effect whatever until several applications of the agent have been made. On the basis

of this restricted evidence no conclusions can be drawn as to any immediate effects upon –SH levels. Calcutt and Coates (1961) have pointed out that prior treatment with dimethylbenzanthracene does appear to affect the timing of a later response to croton oil treatment. This however does not necessarily imply an initial effect upon sulphydryl groups themselves.

When after an initial treatment with a carcinogen further applications of the same agent or of croton oil are made there is a well defined elevation of –SH levels. This suggests that the main response is during the promotion phase. Whether the apparent later decline in –SH levels to below normal values should be regarded as part of this process or as an independent stage is uncertain.

The whole picture in terms of sulphydryl levels can be summarised pictorially as in Fig. 1.

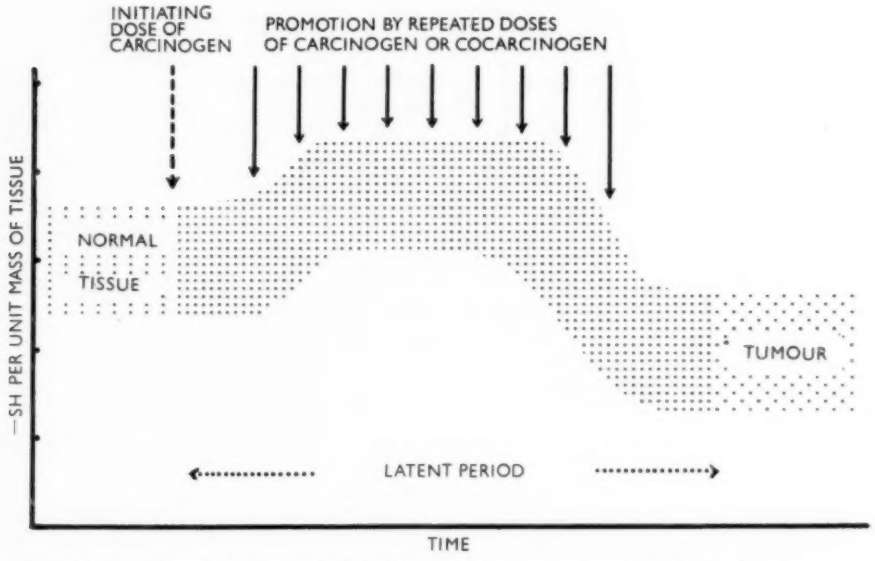

FIG. 1.—Changes in tissue sulphydryl levels during carcinogenesis by chemical agents.

GENERAL DISCUSSION

The evidence which has been quoted above offers a reasonable basis for the overall picture of the behaviour of tissue –SH groups during the carcinogenic process. That there is a direct association between the carcinogenic process and –SH levels is attested by the target tissue specificity of the response to carcinogens and the inhibitory effect of known –SH inhibitors upon the carcinogenic response. Whether the actual tissue sulphydryl content is directly involved in carcinogenesis or whether the –SH level is merely symptomatic of some other more essential tissue change is unknown. Certainly it seems very unlikely that –SH levels could be altered without concomitant changes in enzymatic and other cellular activities. The –SH measurements, however, do offer a direct estimate of the biochemical

status of the tissue from the time of initiation through to the emergence of the developed tumour, and as such offer considerable scope for the further assessment of factors influencing carcinogenesis.

Evidence for the origin of the –SH groups which appear in tissues during the early stages of treatment with carcinogenic chemicals or cocarcinogens is lacking. Again the question as to what happens to this material during the later stages of carcinogenesis still requires an answer.

This paper has collected together data from many and varied sources. Much of this information is superficially unrelated but on the basis of involvement with sulphydryl levels a unifying factor is apparent. The original hypothesis that an elevated sulphydryl level in the target tissue is an essential for tumorigenesis has been found consistent with other data in the literature. At the same time it is suggested that a more complete picture would be one of elevation of tissue –SH levels followed by a fall to subnormal values and the persistence of this latter value into the formed tumour.

No excuse is offered for this paper being speculative in parts; it is merely hoped that this will help to stimulate further experimental work and the publication of data which will help to confirm or refute the ideas which have been expressed.

SUMMARY

1. The evidence for chemical carcinogens causing a rise in target tissue sulphydryl levels has been summarised.

2. It has been shown that many agents or factors promoting or inhibiting carcinogenesis can affect tissue –SH levels in a manner which is predictable on the hypothesis that elevated tissue –SH is an essential part of the carcinogenic process.

3. It is concluded that during carcinogenesis a period of elevated tissue –SH occurs and is followed by a period of subnormal levels. This latter subnormal level is persistent into the resultant tumour.

4. Influence upon sulphydryl levels is a unifying factor which connects much apparently unrelated experimental data.

I am indebted to Mr. D. Doxey of this Department, for permission to quote from his umpublished experimental results.

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REFERENCES

Abels, J. C., Rekers, P. E., Martin, H. and Rhoads, C. P.—(1942) *Cancer Res.*, **2**. 381.

Agate, F. J., Antopol, W., Glaubach, Susi, Agate, Fay and Graff, S.—(1955) *Ibid.*, **15**, 6.

Ahlbohm, H. E.—(1936) *Brit. med. J.*, ii, 331.

Baumann, C. A.—(1948) *J. Amer. diet. Ass.*, **24**, 573.

Berenblum, I.—(1935) *J. Path. Bact.*, **40**, 549.

Berman, C.—(1951) ' Primary Carcinoma of the Liver '. London (H. K. Lewis).

Boyland, E. and Mawson, Elinor, H.—(1938) *Biochem. J.*, **32**, 1460.

Byerrum, R. U., Erway, H. F. and Dubois, K. P.—(1948) *Arch. Biochem.*, **17**, 41.

Calcutt, G.—(1949) *Brit. J. Cancer*, **3**, 306.—(1961*a*) *Ibid.*, **15**, 157.—(1961*b*) *Ibid.*, **15**, 157.—(1961*c*) *Naturwissenschaften*, in press.

UMI

Calcutt, G. and Coates, Joan.—(1961) *Brit. J. Cancer*, **15**, 360.
Idem and Doxey, D.—(1959) *Exp. Cell Res.*, **17**, 542.
Iidem and Coates, Joan.—(1959) *Brit. J. Cancer*, **13**, 711.—(1960) *Ibid.*, **14**, 746.—(1961*a*) *Ibid.*, **15**, 149.—(1961*b*) *Nature*, **192**, 164.
Carruthers, G.—(1940) *Arch. Path.*, **30**, 1184.
Crabtree, H. G.—(1944) *Cancer Res.*, **4**, 688.—(1945) *Ibid.*, **5**, 346.—(1946) *Ibid.*, **6**, 553.—(1948) *Brit. J. Cancer*, **2**, 281.
Di Paolo, J. A. and Niedbala, T. F.—(1957) *Proc. Soc. exp. Biol. N.Y.*, **96**, 255.
Fiala, S. and Fiala, Anna E.—(1959) *Brit. J. Cancer*, **13**, 136.
Flesch, P. and Goldstone, S. B.—(1952) *J. invest. Derm.*, **18**, 267.
Gallico, E. and Boretti, G.—(1948) *Tumori*, **34**, 130.
Garzia, A. and Dansi, A.—(1953) *Il Pharmaco*, **8**, 449.
Goss, H. and Gregory, P. W.—(1935) *Proc. Soc. exp. Biol. N.Y.*, **32**, 681.
Greenstein, J. P. and Leuthardt, F. M.—(1945) *J. nat. Cancer Inst.*, **5**, 111.
Gregory, P. W. and Goss, H.—(1934) *J. exp. Zool.*, **69**, 13.
Iki, H.—(1939) *Gann*, **33**, 216, quoted by Cook, J. W. and Kennaway, E. L.—(1940) *Amer. J. Cancer*, **39**, 381.
Kaunitz, H., Johnson, R. E. and Slanetz, C. A.—(1952) *J. Nutr.*, **46**, 151.
Kinosita, R.—(1938) *J. Japan Gastroenterol. Soc.*, **37**, 513, quoted by Greenstein J. P.—(1954) ' Biochemistry of Cancer '. New York (Academic Press).
Kline, B. E., Miller, J. A., Rusch, H. P. and Baumann, C. A.—(1946) *Cancer Res.*, **6**, 5.
Kortweg, R. and Thomas, F.—(1939) *Amer. J. Cancer*, **37**, 36.
Lazarow, A.—(1954) in ' Glutathione, a Symposium '. New York (Academic Press).
Lusky, L. M., Braun, H. A. and Woodard, G.—(1947) *Cancer Res.*, **7**, 667.
Masayama, I., Iki, H., Yokayama, T. and Hasimoto, M.—(1938) *Gann*, **32**, 303, quoted by Cook, J. W. and Kennaway, E. L.—(1940) *Amer. J. Cancer*, **39**, 381.
Moon, H. D., Li, C. H. and Simpson, Miriam, E.—(1956) *Cancer Res.*, **16**, 111.
Idem and Simpson, Miriam, E.—(1955) *Ibid.*, **15**, 403.
Noble, R. L. and Walters, J. H.—(1954) *Proc. Amer. Ass. Cancer Res.*, **1**, 35.
O'Neal, M. A., Hoffmann, H. E., Dodge, B. G. and Griffin, A. C.—(1958) *J. nat. Cancer Inst.*, **21**, 1161.
Pelc, S. R. and Fell, Honor, B.—(1960) *Exp. Cell Res.*, **19**, 99.
Reimann, S. P. and Hall, E. M.—(1936) *Arch. Path.*, **22**, 55.
Riley, J. F. and Pettigrew, F.—(1944) *Cancer Res.*, **4**, 502.
Idem and Wallace, A. B.—(1941) *Brit. J. exp. Path.*, **22**, 24.
Robertson, C. H., O'Neal, M. A., Griffin, A. C. and Richardson, H. L.—(1953) *Cancer Res.*, **13**, 776.
Rondoni, P. and Boretti, G.—(1947) *Tumori*, **33**, 274.
Rosicky, J. and Hatschek, R.—(1943) *Z. Krebsforsch.*, **54**, 26.
Schubert, M. P.—(1936) *J. biol. Chem.*, **114**, 341.
Schulze, G.—(1960) *Z. ges. exp. Med.*, **133**, 194.
Setälä, K., Äyräpää, O., Niskanen, E. E., Stjernvall, L. and Nyholm, M.—(1960) *Naturwissenschaften*, **47**, 357.
Stocks, P. and Karn, Mary N.—(1933) *Ann. Eugen., Lond.*, **5**, 237.
Tannenbaum, A.—(1944) *Cancer Res.*, **4**, 683.
Idem and Silverstone, H.—(1957) Chapter 9 in ' Cancer '. Edited by Raven. London (R. W. Butterworth).
Wood, J. L. and Kraynak, M. E.—(1953) *Cancer Res.*, **13**, 358.

SULPHYDRYL LEVELS OF THE LIVER AND KIDNEYS FROM RATS FED DL. ETHIONINE

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A STUDY of the variations in sulphydryl (–SH) levels in target and non-target tissues subjected to chemical carcinogens by Calcutt, Doxey and Coates (1960, 1961) has led to the hypothesis that an elevation of target tissue –SH levels is an essential part of the tumour induction process. Further evidence in favour of this hypothesis has been found experimentally by Calcutt and Coates (1961). As an extension of this work consideration has also been given to the case of DL. ethionine as an agent capable of inducing liver tumours in female rats. This activity was first described by Popper, de la Huerga and Yesinick (1953).

In the present work changes in liver (susceptible tissue) and kidney (non-susceptible tissue) –SH levels over a period of ten weeks from the commencement of treatment have been examined in relation to levels in untreated control animals. Additionally samples from the livers of both control and treated animals have been examined histologically throughout the period of the experiment to see whether any detectable histologic changes can be correlated with the biochemical findings.

METHODS

The animals used were female Wistar rats aged twelve weeks at the commencement of the experiment. Ten were used as controls and fourteen as experimental animals. Ethionine was added at the rate of 0·2 per cent to powdered cubed diet and the resultant mixture made to a damp mash with water, and fed *ad libitum* to the experimental animals. The control animals received a similarly prepared diet, but without the ethionine.

Sulphydryl estimations were carried out by the technique described by Calcutt and Doxy (1959) and Calcutt *et al.* (1960). This method gives a total value for glutathione and tissue protein bound sulphydryl.

For histological purposes small pieces of tissue were fixed in formol-saline, embedded in paraffin wax and cut at a thickness of 5 μ. Sections were stained with haematoxylin and eosin.

RESULTS

Effects on –SH levels

Findings in respect of –SH levels in the liver are given in Fig. 1. The control level values showed no indications of any pattern, so a mean value and standard deviation have been calculated. From the second day of treatment through to the end of the experiment, the experimental liver –SH values were consistently much higher than the control values.

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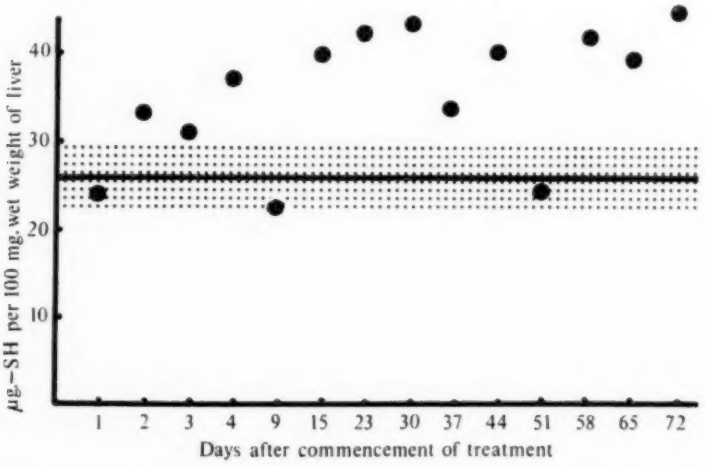


FIG. 1.——SH changes in rat liver during feeding with ethionine. The mean control value is shown as a heavy line and the standard deviation by the dotted area. Experimental values are indicated as filled circles.

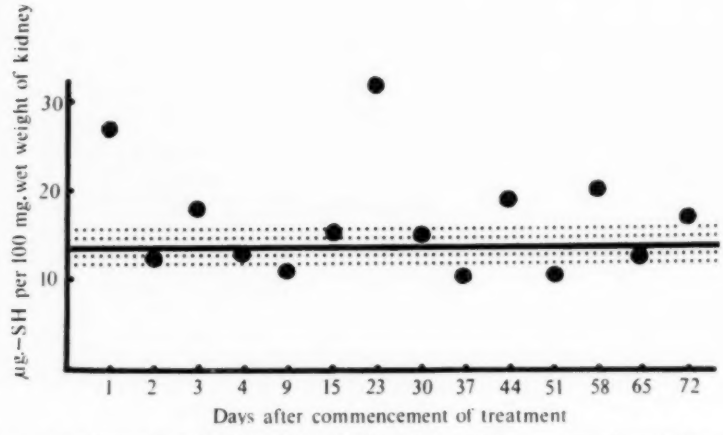
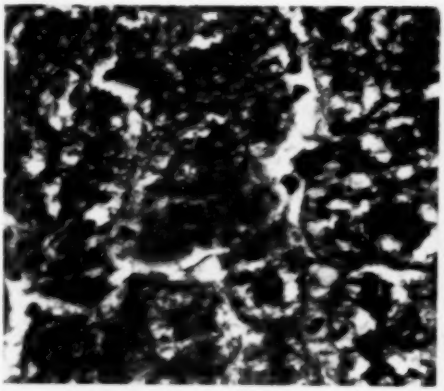


FIG. 2.——SH changes in rat kidney during feeding with ethionine. Symbols as in Fig. 1.

EXPLANATION OF PLATE

FIG. 3.—Section from control rat liver. ×550.
FIG. 4.—Section from rat liver after 9 days on ethionine. ×550.
FIG. 5.—Section from rat liver after 30 days on ethionine. ×550.
FIG. 6.—Section from rat liver after 65 days on ethionine. ×550.



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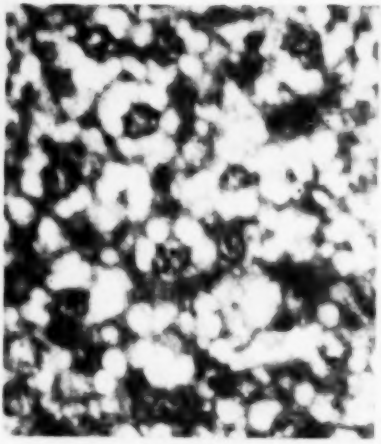

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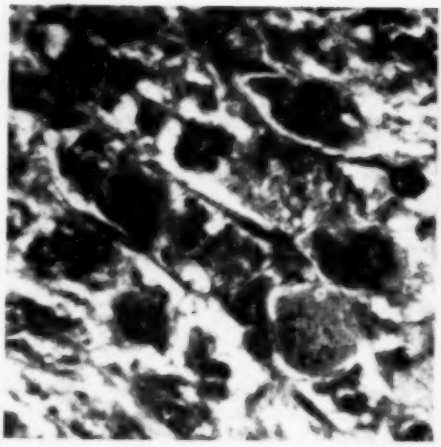

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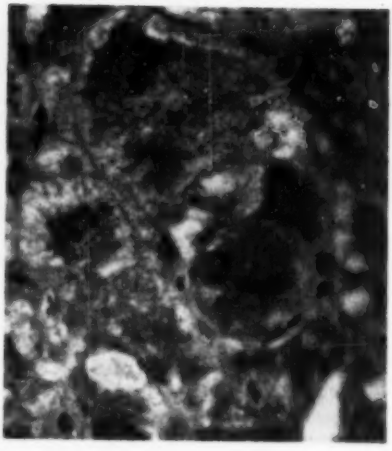

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The results of the measurements of kidney–SH values are shown in Fig. 2. Here again a mean value and standard deviation have been calculated in respect of the control figures. With two noticeable exceptions the experimental figures show no particular deviation from the control levels.

Histological changes in the livers

The sections taken from control livers have shown nothing unusual. A certain amount of fat was present, but mitotic figures were rare—Fig. 3.

The findings in respect of the sections from the experimental livers are summarised below :

4 days—indications of fat accumulation.
9 days—intense loading of cells with fat (Fig. 4).
15 days—some cells much enlarged and showing denser cytoplasm.
23 days—most fat deposits now gone and cells showing dense cytoplasm. An occasional mitosis present, usually in enlarged cells.
30 days—many mitoses in large cells (Fig. 5).
37 days—similar to previous section.
44 days—few mitoses, some fatty infiltration.
51 days—no mitoses found but increased fat.
58 days—less fat and dense cytoplasm in some cells.
65 days—frequent mitoses—usually in large cells. Little fat present (Fig. 6).
72 days—very similar to the previous section.

Where mitoses have been present approximately 20 per cent of these have been atypical. Abnormalities have been various and found at all stages of the mitotic cycle.

DISCUSSION

The changes in liver sulphydryl values are completely in keeping with the previous work by Calcutt *et al.* (1960, 1961) and can be regarded as further evidence in favour of the hypothesis of an increased tissue –SH level during tumour induction. In the case of the kidney the results are suggestive of the carcinogenic agent having little or no effect. No explanation is available for the two kidneys with very high –SH values, but this does not exclude the possibility of disease being responsible. Equally there is nothing to exclude these results being the direct consequence of the ethionine feeding. This, however, seems unlikely in view of there only being the two isolated cases.

The histological changes encountered during the first few weeks of the experiment are similar to those found by other workers and summarised by Popper *et al.* (1953). The appearance of a second cycle of—fat infiltration, loss of fat and mitosis again—does not appear to have been described previously. As the ethionine feeding was continuous throughout the experiment it is difficult to account for this apparent repetition of events. Obviously, further experiments are called for to see whether this apparent repetitive cycle occurs again over longer periods.

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The fact that mitosis occurs when the liver –SH is high, is interesting in view of the extensive consideration which has been given to the question of sulphydryl groups in relation to cell division (see summary by Needham (1950)). Otherwise, at the moment there would appear to be little correlation between the liver –SH levels and the histologic picture.

SUMMARY

1. The feeding of 0·2 per cent ethionine in the diet to female rats, was found to cause prolonged elevations of liver –SH levels, but to have little effect on kidney –SH levels.

2. Histologically the livers showed a cycle of fatty infiltration, loss of fat and mitosis. This occurred over the period 1–5 weeks and again at 6–10 weeks after commencement of the experiment.

The expenses of this work were defrayed from a block grant from the British Empire Cancer Campaign.

REFERENCES

CALCUTT, G. AND COATES, JOAN.—(1961) *Brit. J. Cancer*, **15**, 360.
Idem AND DOXEY, D.—(1959) *Exp. Cell. Res.*, **17**, 542.
Idem AND COATES, JOAN.—(1960) *Brit. J. Cancer*, **14**, 749.—(1961) *Ibid.*, **15**, 149.
NEEDHAM, J.—(1950) 'Biochemistry and Morphogenesis'. Cambridge (Cambridge University Press).
POPPER, H., DE LA HUERGA, J. AND YESINICK, C.—(1953) *Science*, **118**, 80.

THE EFFECT OF NORMAL AND MALIGNANT CHROMOSOMAL MATERIAL ON NEWBORN RATS

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MANY attempts to produce malignant changes in animals by injecting them with different components of normal and malignant cells have been made during recent years. Some were successful, others failed completely. The picture drawn by the results of these experiments points to two possible alternatives. Either ribonucleic acid (RNA) as a constituent of some virus-like agent is responsible for carcinogenesis or deoxyribonucleic acid (DNA) as a carrier of genetic information. Meek and Hewer (1959) succeeded in producing carcinomas in newborn mice using herring sperm DNA, but they do not ascribe the tumour production unequivocally to DNA because some of their other DNA preparations were not active. Stolk (1960) produced neoplasms in fish by the same method and DiMayorca, Eddy, Stewart, Hunter, Friend and Bendich (1959) using the technique of cultivation *in vitro* prepared DNA from SE polyoma virus by extraction with phenol or *p*-aminosalicylate. Both preparations were cytopathogenic in mouse embryos in tissue cultures and carcinogenic in hamsters. This " infective DNA " was easily inactivated by the action of DNA-ase, but was quite resistant to RNA-ase.

Assuming that the successful production of tumours in mice and fish with DNA might have a character of transformations, newborn rats were injected with histone, deoxyribonucleoprotein (DNP) and deoxyribonucleic acid (DNA), prepared from calf thymus and from spleen and liver of rats bearing acute myeloid leukaemia. All three mentioned substances are constituents of mammalian chromosomes.

MATERIALS AND METHODS

Histones, DNP and DNA were prepared from calf thymus and from spleen and liver of leukaemic rats infiltrated heavily with leukaemic leucocytes (Hlavayova, 1957).

The tissue was washed with 0·14 M NaCl containing 0·01 M sodium citrate by blending in Waring Blendor and spinning at 900 *g* for 20 minutes. After 4–6 washings the final sediment was extracted with 0·2 N HCl for histone or dissolved in 1 M NaCl containing 0·01 M sodium citrate.

The acid extract containing histone was dialysed against distilled water and lyophilised.

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The viscous DNP solution was clarified by centrifugation at 16,000 *g* for 20 minutes and precipitated by dilution with 5 volumes of 0·01 M sodium citrate. The fibrous precipitate was homogenized in physiological saline for injection, or redissolved in 1 M NaCl 0·01 M sodium citrate for preparation of DNA.

DNA was prepared by deproteinization of the viscous DNP solution according to Sevag, Lackman and Smolens (1938).

Animals in litters containing an average of ten animals were injected according to the following scheme :

Group 1 A : 0·2 ml. of 4 or 0·6 per cent solution of calf thymus histone.
B : 0·2 ml. of 4 or 0·6 per cent solution of leukaemic rat histone.
Group 2 A : 0·2 ml. of DNP homogenate (calf thymus).
B : 0·2 ml. of DNP homogenate (leukaemic rats).
Group 3 A : 0·2 ml. of 4 or 0·6 per cent solution of calf thymus DNA.
B : 0·2 ml. of 4 or 0·6 per cent solution of leukaemic rat DNA.
Group 4 : 0·2 ml. of physiological saline.
Group 5 A : 0·2 ml. of 4 per cent solution of calf thymus DNA
B : 0·2 ml. of 4 per cent solution of leukaemic rat DNA.

Each subgroup (capital letters) represents ten litters. The DNP homogenate contained 3–4 mg. of DNP in 0·2 ml. according to the nitrogen and phosphorus estimation. All histones and DNAs were dissolved in physiological saline. All injections were placed in dorsal subcutis within 16 hours of birth and animals in groups 1–4 were injected once only, animals in the group 5 were injected each alternative day for three weeks and observed for 14 months. Animals in groups 1–4 were killed at two day intervals beginning the second day after birth. For each animal killed smears of blood and bone marrow as well as histological sections from kidney, heart, lungs, liver, spleen and thymus were examined. All the animals were weighed in groups each alternate day.

RESULTS

All the preparations of histones were toxic, resulting in high mortality in the newborn rats during the period of 5–7 days after injection. It was impossible to estimate the L.D. 50 for newborn rats, but, for example, after injection of 0·1 ml. of 4 per cent calf thymus histone solution 29 per cent and after injection of 0·2 ml. of the same solution, 42 per cent of rats died within 5–7 days. The toxicity of histones prepared from spleen and liver of leukaemic rats was much higher, e.g. 72 per cent of animals died after injection of 0·2 ml. of 4 per cent solution. All the DNP preparations were nearly as toxic as histones.

Slight increases of leucocytes and more than 300 per cent increases of erythroblasts in blood and bone marrow were observed after injection of histones or DNPs. Seven days after injection the leucocyte count was normal but the erythroblasts were still increased by about 200 per cent. Two weeks after injection of histone or DNP the figures were normal. Except for some degenerative changes in liver no other histological changes in the examined tissues could be seen.

Within ten days of injection of histone or DNP sterile encapsulated abscesses arose at the site of injection. All healed spontaneously.

Animals treated with histone or DNP were significantly retarded in growth and development (Figs. 1 and 2). Their resistance to infections was low and all died within four months of injection.

No changes in blood, bone marrow, in histologically examined tissues or in the growth of animals injected with DNA were observed.

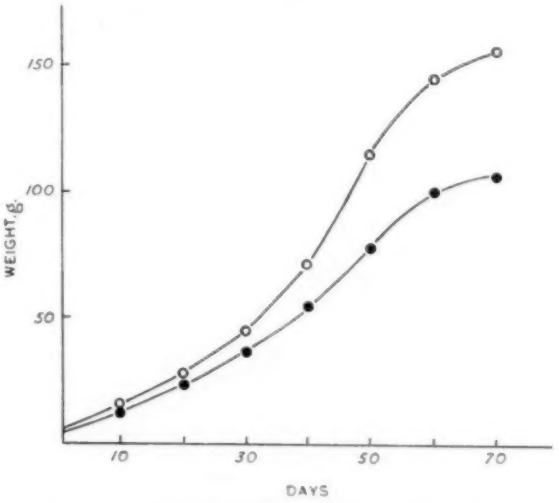


FIG. 1.—Weight averages of normal and treated rats.
—○— Controls and rats injected with DNA
—●— Rats injected with calf thymus histone (4 per cent solution, 0·2 ml.).

Neither histone, DNP nor DNA from leukaemic rats caused malignant changes during the observation period (four months for histone, 14 months for DNP survivals, for DNA and controls).

DISCUSSION

It is beyond doubt that the chromosomes contain the entire genetic apparatus of the cell. While the role of DNA in coding genetical information seems to be established, the function of at least two groups of proteins accompanying DNA in chromosomes is not clear. The major group represented by histones, proteins with iso-electric point at pH 10–11 have, according to Danielli (1950), because of their high positive electric charge, the maximal possibility of reacting with nucleic acids and thus of controlling the rate of metabolic activity.

In experiments *in vitro* (Becker and Green, 1960; Sandritter, Fischer, Süssenberger and Schiemer, 1959; Stedman, Stedman and Pettigrew, 1944) and *in vivo* (Zbarskii and Perevozhchikova, 1944) the histones showed high toxicity damaging the cell RNA and DNA by active penetration of cellular membranes and interaction resulting in insoluble precipitate. This mechanism could explain the action of histone in newborn rats. The biosynthetic pathways important for the early stage of the postnatal development were damaged in injected animals

probably by the interaction of injected histones with nucleic acids—especially RNA. As a result there was a significant retardation of growth. The mechanism of this action is as yet unknown but the recent discovery of histone-like proteins in cytoplasmic particles (Butler, Cohn and Simson, 1960; Setterfield, Neelin, Neelin and Bayley, 1960; Waller and Harris, 1961) which seem to have some RNA-ase activity Leslie, 1961) might point to the importance of these proteins in the mechanism of biosynthesis.

Riman and Vesely (1957) have described retardation of growth, leukocytosis, reticulocytosis and deformities in joints of all extremities after injection of DNP preparations from liver of leukaemic mice into newborn rats. According to our results, at least a part of these changes could not be due to the specific action of leukaemic DNP, but these are caused by the toxic effect of histone present in injected DNP.

The fact that no malignant changes in animals treated with histone, DNP or DNA were observed seems to suggest that the production of tumours by the normal or leukaemic chromosome constituents is very difficult if not impossible. Lieder (1960) described a similar failure of specific DNA to produce neoplastic changes in fish and raised the question if the carcinogenetic effect of herring sperm DNA is not due to some substance present in this DNA only.

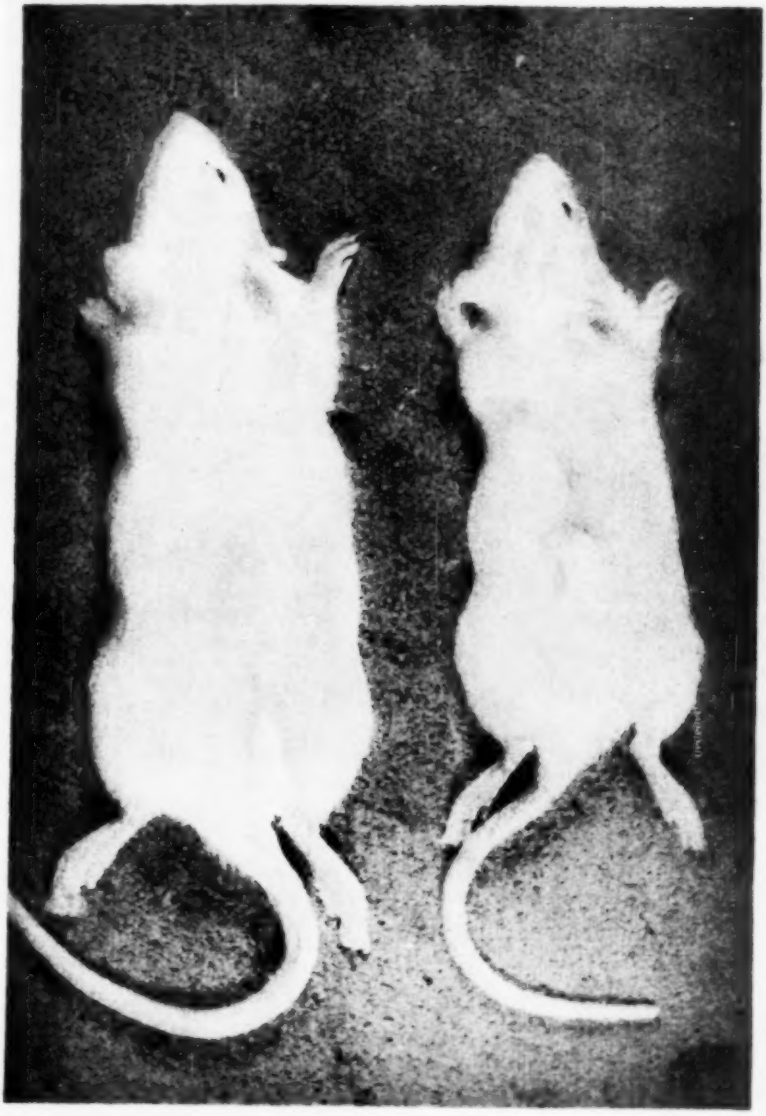
Thiery (1950) obtained histiocytomas after treatment of young dogs with crude DNP preparations made from the Sticker sarcoma and Stasney, Cantarow and Paschkis (1950) succeeded in inducing sarcomas and leukaemias in rats by injecting the chromatin material from Murphy rat lymphosarcoma. Vallardares (1960) recently described leukaemias in mice injected with nucleoprotein prepared from the Ehrlich ascites tumour. On the other hand similar experiments carried out by Tourtellotte and Storer (1950) with subcellular components, including the nuclear material of the Walker carcinoma, failed to produce neoplastic growth.

According to some recent papers (deCarvalho, Rand and Meyer, 1960; Graffi and Fritz, 1960; Lacour, Lacour, Harel and Huppert, 1960; Latarjet, Rebeyrotte and Moustacchi, 1959) and to our unpublished results, RNA prepared from neoplastic tissue has a significant carcinogenic activity. The method of preparation of DNP and chromatin does not exclude the possibility of contamination with RNA. Even the possibility of the action of either DNA or RNA as described by Graffi and Fritz (1960) for polyoma virus should be considered.

It is of some interest that the histone treated rats strongly resembled the picture of the irradiation disease and especially that of the "runt disease" following the heterotransplantation of tissue in young animals (Amiel and Mathe, 1960; Billingham and Brent, 1957; Simonsen, 1957; Woodruff and Sparrow, 1957). In our opinion at least a part of these symptoms is caused by the toxicity of histones released from the nuclei during the period of massive destruction of cells damaged by the radiation or by the antigenic response of the body.

EXPLANATION OF PLATE.

FIG. 2.—The effect of histone in postnatal development.
Left: control rat.
Right: rat injected at birth with 0·2 ml. of 4 per cent calf thymus histone solution.
Both rats 3 months after treatment. The scar on the back of the treated rat is a healed abscess.



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SUMMARY

The action of histone, deoxyribonucleoprotein and deoxyribonucleic acid prepared from leukaemic rat liver and spleen and from calf thymus was followed in newborn rats. Histones and deoxyribonucleoproteins were toxic, causing erythroblastosis and retardation of growth in injected animals. None of the injected substances caused malignant changes within the observation period of 4 and 14 months respectively.

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REFERENCES

AMIEL, J. L. AND MATHE, G.—(1960) *Experientia*, **16**, 563.
BECKER, F. F. AND GREEN, H.—(1960) *Expt. Cell. Res.*, **19**, 361.
BILLINGHAM, R. E. AND BRENT, L.—(1957) *Transpl. Bull.*, **4**, 67.
BUTLER, J. A. V., COHN, P. AND SIMSON, P.—(1960) *Biochim. biophys. Acta.*, **38**, 386.
DECARVALHO, S., RAND, H. J. AND MEYER, D. P.—(1960) *J. Lab. clin. Med.*, **55**, 706.
DANIELLI, J. F.—(1950) *Cold Spr. Harb. Symp. quant. Biol.*, **14**, 32.
DIMAYORCA, G. A., EDDY, B. E., STEWART, S. E., HUNTER, W. S., FRIEND, C. AND BENDICH, A.—(1959) *Proc. nat. Acad. Sci., Wash.*, **45**, 1805.
GRAFFI, A., FRITZ, D.—(1960) *Rev. franç. clin. biol.*, **5**, 388.
HLAVAYOVA, E.—(1957) *Neoplasma*, **4**, 10.
LACOUR, F., LACOUR, J., HAREL, J., HUPPERT, J.—(1960) *J. nat. Cancer Inst.*, **24**, 301.
LATARJET, R., REBEYROTTE, N., MOUSTACCHI, E.—(1959) *Haematologica Latina*, **2**. Suppl. II, 13.
LESLIE, E.—(1961) *Nature, Lond.*, **189**, 260.
LIEDER, N.—(1960) *Naturwissenschaften*, **47**, 308.
MEEK, E. S., HEWER, T. F.—(1959) *Brit. J. Cancer*, **13**, 121.
RIMAN, J., VESELY, J.—(1957) *Chem. Listy*, **51**, 1954.
SANDRITTER, W., FISCHER, H., SÜSSENBERGER, K., SCHIEMER, H. G.—(1959) *Exp. Cell Res.*, **17**, 197.
SETTERFIELD, G., NEELIN, J. M., NEELIN, E. M., BAYLEY, S. T.—(1960) *J. mol. Biol.*, **2**, 416.
SEVAG, M. G., LACKMAN, D. B., SMOLENS, J.—(1938) *J. biol. Chem.*, **124**, 425.
SIMONSEN, M.—(1957) *Acta path. microbiol. scand.*, **40**, 480.
STASNEY, J., CANTAROW, A., PASCHKIS, K. E.—(1950) *Cancer Res.*, **10**, 775.
STEDMAN, E., STEDMAN, E., PETTIGREW, F.—(1944) *Biochem. J.*, **38**, XXXI.
STOLK, A.—(1960) *Naturwissenschaften*, **47**, 88.
THIERY, C.—(1950) *C.R. Soc. Biol. Paris*, **144**, 745.
TOURTELLOTTE, W. W., STORER, J. B.—(1950) *Cancer Res.*, **10**, 783.
VALLADARES, V.—(1960) *Med. expt.*, **2**, 309.
WALLER, J. P., HARRIS, J. I.—(1961) *Proc. nat. Acad. Sci. Wash.*, **47**, 18.
WOODRUFF, M. F. A., SPARROW, M.—(1957) *Transpl. Bull.*, **4**, 157.
ZBARSKI, I. B., PEREVOZHCHIKOVA, K. A.—(1944) *Byull. eksp. biol. med.* (U.S.S.R.), **38**, 61.

MULTIPLE MYELOMA—AN *IN VITRO* STUDY

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RECENT experimental investigations of plasma cell myeloma have been directed mainly towards the elucidation of problems of protein synthesis in the myeloma cell (Nathan, Fahey and Potter, 1958; Vasquez, 1958; Truax, Bray and Perry 1961), and the effects of chemotherapy on tumour progression *in vivo* (Kay, Ledlie and Sbresni, 1959; Denman and Ward, 1960; Benefiel, Helsper and Sharp, 1960; Hayes, Spurr and Hines, 1961). Morphological studies have been concerned with details of ultrastructure as revealed by the electron microscope (Braunsteiner, Fellinger and Pakesch, 1957; Dalton, Potter and Merwin, 1961). In our present work we have investigated the dynamic morphology of myeloma cells, using phase contrast optics and time-lapse cinematography. We have been able to correlate these findings with similar work on the rat plasma cell carried out by Thiéry (1960) and personal observations on normal rat plasma cells.

The myeloma cells have been exposed *in vitro* to a recently developed chemotherapeutic compound, 1-aminocyclopentanecarboxylic acid (NSC-1026), which has proved useful in the treatment of multiple myelomatosis (Benefiel *et al.*, 1960) at a total dose level of 400–800 mg./kg. body weight. At the same time, preliminary work has been carried out on the chromosomal pattern of growing peripheral blood cells of myeloma patients. While no "marker" chromosomes were detected in our material, it is interesting to note that chromosomal aberrations have recently been established in sternal marrow preparations from a related disease, Waldenström's macroglobulinaemia (Bottura, Ferrari and Veiga, 1961). Samples of growing peripheral blood cells were also investigated before and after a course of NSC-1026.

MATERIALS AND TECHNIQUES

Material for examination was obtained from three cases of multiple myeloma.

Case 1.—M.L. Female, 55 years. She complained of a lump ($5 \times 3 \times 3$ cm.) on the right side of the head. The tumour had been growing over a period of five months. X-ray demonstrated a destructive process in the underlying bone and histological examination of a biopsy showed the typical appearance of myeloma. Specimens of the tumour, bone marrow and peripheral blood were taken for study *in vitro*. The next day, the patient received the first of 10 daily intravenous injections of NSC-1026. Two days after the last injection repeat samples of blood, bone marrow and tumour were obtained.

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Case 2.—W.M. Male, 70 years. There was a 14 month history of bone pain diagnosed elsewhere as due to multiple myeloma on the basis of X-ray findings and marrow biopsy. Eight months ago he received 17 X-ray treatments to the thoracic spine and later was started on a course of urethane which had to be discontinued after a month on account of nausea. On examination he was an emaciated, ill subject. Samples of blood and bone marrow were taken for culture (bone marrow smears confirmed the diagnosis). Similar material was obtained five days after completing a course of NSC-1026 therapy.

Case 3.—N.R. Female, 63 years. Four years ago a complaint of aching bones led to bone marrow biopsy which established the diagnosis of multiple myeloma. A trial of urethane at this time was discontinued on account of leucopenia. She has remained remarkably well since then, although X-rays show extensive osteolytic lesions in the skeleton. Recent exacerbation of bone pain led to a trial of NSC-1026 therapy. Specimens of blood and bone marrow were obtained before commencing therapy.

Culture techniques

Explants of tumour, derived from Case 1, were grown (a) under narrow cellophane strips in modified Rose chambers (Rose *et al.*, 1958) using Eagle's medium enriched by 10 per cent human serum, (b) on plasma clot in T30 flasks, using 10 per cent horse serum in Eagle's medium. Subculture was carried out by trypsinization (0·2 per cent trypsin (Nutritional Biochemicals Corporation) for 10 minutes) and transfer to fresh plasma clot.

Twenty-five to 40 ml. samples of venous blood were collected in tubes, and cultures of the buffy coat were prepared, using bactophytohemagglutinin (Difco) according to techniques already established (Hungerford *et al.*, 1959; Nowell, 1960; Ohnuki, Awa and Pomerat, 1962). Material from all three patients was was available for study but owing to a temporary change in the character of bactophytohemagglutinin, comparable samples of blood before and after NSC-1026 therapy were successfully grown only in Case 1.

Explants of bone marrow were grown variously; (a) in Rose chambers under cellophane; (b) on plasma clot in T30 flasks—both as described for tumor explants; (c) on glass, in T30 flasks, using Eagle's medium, with 10 per cent horse serum, for chromosome studies: after 18 hours culture the cells were exposed to colchicine (0·05 ml. of 1/10,000 dilution to 5 ml. of medium) for three hours before squashing and microscopic examination; (d) heparinized samples were grown according to the buffy coat technique for peripheral blood.

RESULTS

Behaviour of myeloma cells in tissue culture

The outgrowth from bone marrow and tumour explants always contained an admixture of other blood elements and fibroblasts, in addition to the myeloma cells. The myeloma cells were identified using phase contrast optics, by their typical morphology and cytoplasmic movements, correlated with experience gained in examination before and after fixation and staining.

Cinematographic recordings were made of myeloma cells and their dynamic behaviour was subsequently analysed, making particular use of the Vanguard

analyser which allows viewing of enlarged pictures of individual film frames at any desired speed. Four different types of cell movement were observed. (1) A lesser or greater part of the cell membrane may show a fine undulating activity. Some cells have shown a unusually marked membrane undulation (Fig. 1) but in general this cell movement is only noticeable when the process is speeded up by time-lapse cinematography. Thiéry (1960), working on rat plasma cells has stressed that it is the plasmablast which is characterized by undulation of the cell membrane, which was not observed by him in the mature plasma cell. Almost all myeloma cells, in our studies, have shown this type of activity. (2) At one or more sites on the cell membrane, a thick pulsating extension of cytoplasm may be formed (Fig. 7, 8, 9). These pseudopodia extend and retract rapidly, taking about 30 seconds to complete the cycle, and showing a period of rest (usually about 30 seconds) between successive pulsations at the same site on the cell membrane. At the tip of the pseudopodium, there is an active undulation of the membrane and secondary protrusions of fine processes may also be seen in this area (Fig. 2).

EXPLANATION OF PLATES

All the phase contrast microphotographs were taken on living cells, except Fig. 23–26 inclusive.

FIG. 1.—Myeloma cell showing remarkable undulation of the cell membrane. Phase Contrast. ×2000.

FIG. 2.—Myeloma cell exhibiting two short pseudopodal extensions of the cytoplasm; fine cytoplasmic processes are seen at the tips of the pseudopods and elsewhere on the cell margin. P.C. ×2600.

FIG. 3.—Myeloma cell showing grossly eccentric position of the nucleus. P.C. ×2600.

FIG. 4.—Myeloma cell which showed fine undulating activity of the margin at the left lower pole. P.C. ×2600.

FIG. 5.—Same cell as Fig. 4, showing broad pseudopodal extension of the cytoplasm at the site of undulation. P.C. ×2600.

FIG. 6.—The fine network seen in the cytoplasm of this degenerating myeloma cell is due to vacuolisation of the endoplasmic reticulum. (The large white area to the left of the nucleus is an artefact.) P.C. ×2600.

FIG. 7, 8, 9.—Rapid cytoplasmic pulsation in myeloma cells. Same field photographed at 0, 14, 35 seconds. P.C. ×900 approximately.

FIG. 10, 11, 12.—Division of myeloma cell showing metaphase, anaphase and telophase respectively. P.C. ×1000 approximately.

FIG. 13.—Untreated explant of myelomatous tumour (left half of field) with healthy outgrowth of myeloma cells and fibroblasts. May-Grünwald-Giesma. ×310.

FIG. 14.—Myeloma explant treated for 30 hours with 1 per cent NSC-1026. Comparable field to that in Fig. 13. Degenerative changes are seen in the cells of the outgrowth and in the original explant. M.G.G. ×310.

FIG. 15.—Myeloma cells after 15 days *in vitro*. Prominent Golgi areas are seen, there is considerable variation in cell shape, and nuclei are often grossly eccentric. Binucleate and multinucleate forms are present. M.G.G. ×785.

FIG. 16.—Two spindle-shaped myeloma cells are seen in the upper part of the photograph. These may be compared with two lighter-stained fibroblasts, in the middle and left lower parts of the field. Fifteen days *in vitro*. M.G.G. ×785.

FIG. 17–22.—Same field photographed over a period of 5 hours. One myeloma cell (arrowed) shows irregular elongation and contraction, leading finally to cell death (Fig. 22). Other cells in the field show similar spasmodic elongation and contraction, in lesser degree. Treated with 0·5 per cent NSC-1026. P.C. ×750 approximately.

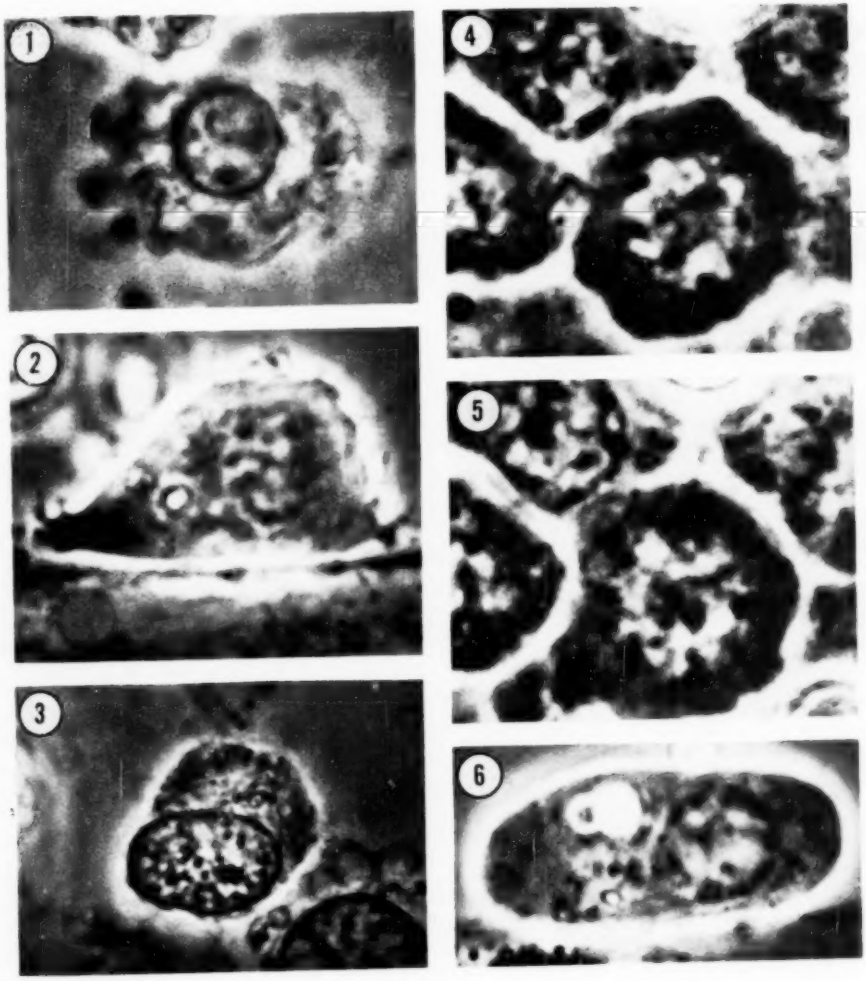
FIG. 23–26. Squash preparations of metaphase plates in buffy coat cultures from Case 1 after treatment with NSC-1026. All stained by acetic-orcein and photographed with phase-contrast optics.

FIG. 23.—Normal metaphase.

FIG. 24.—One example of abnormal chromosome morphology. The idiogram of this cell is seen in Fig. 28.

FIG. 25.—Translocation of chromosomes.

FIG. 26.—Chromosome showing breakage.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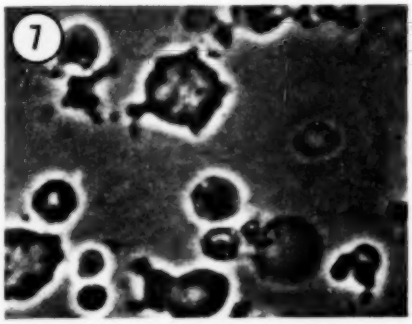
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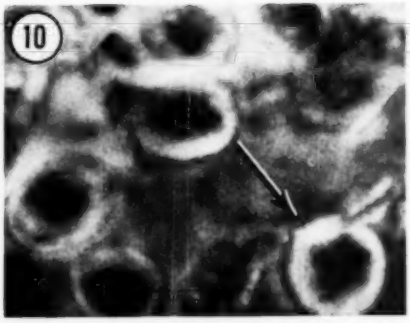
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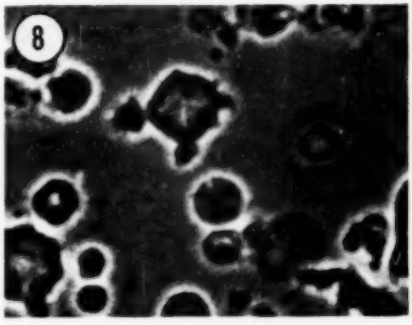
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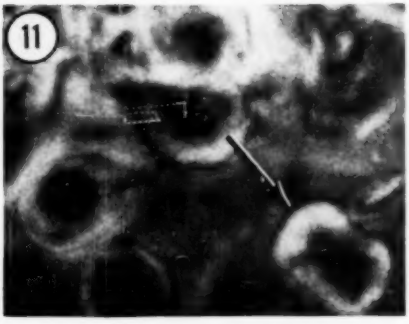
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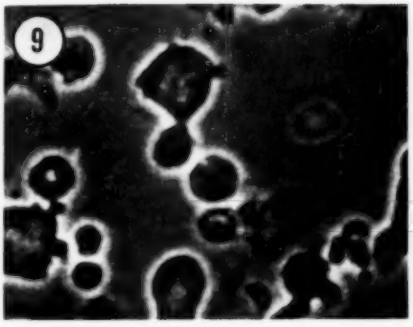
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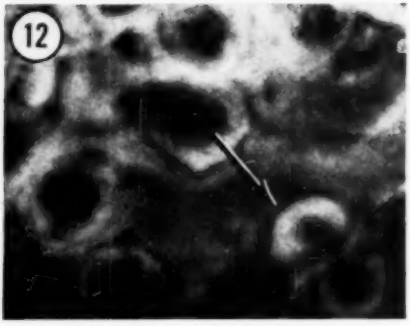
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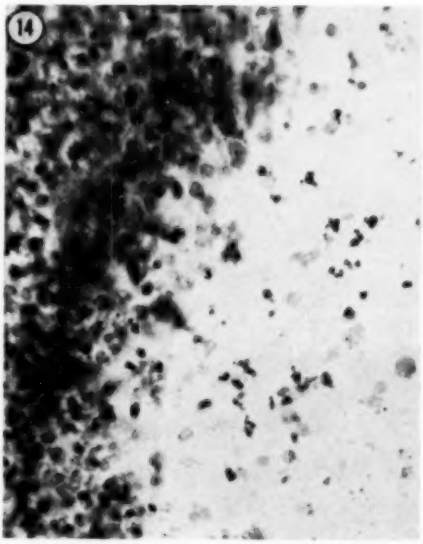

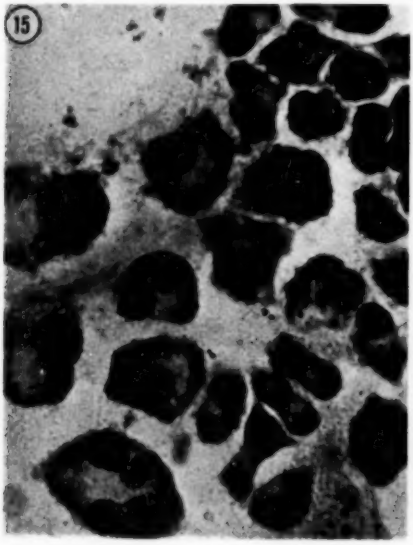

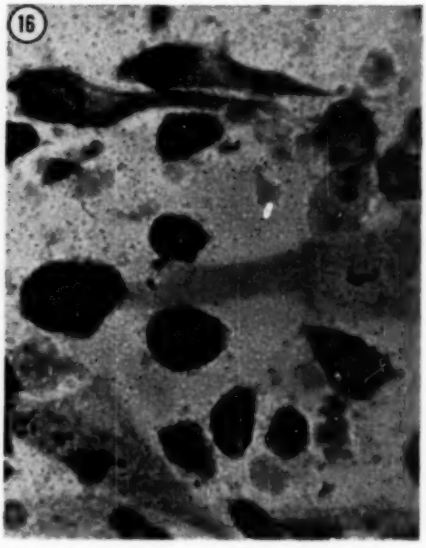

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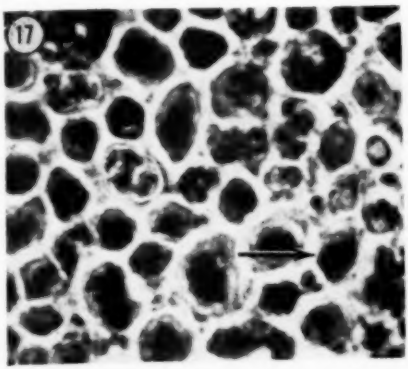
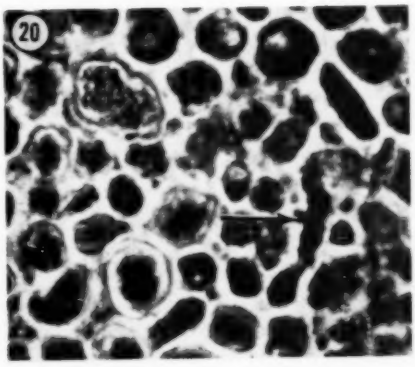
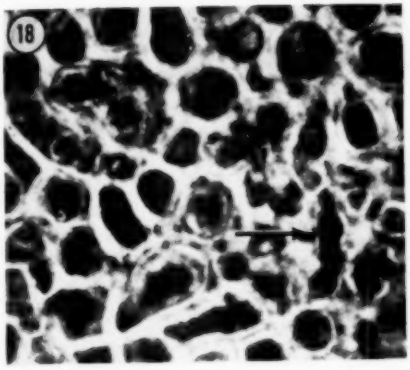
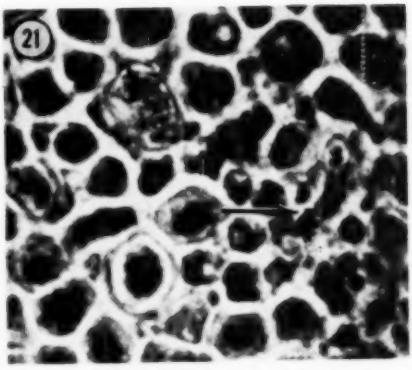
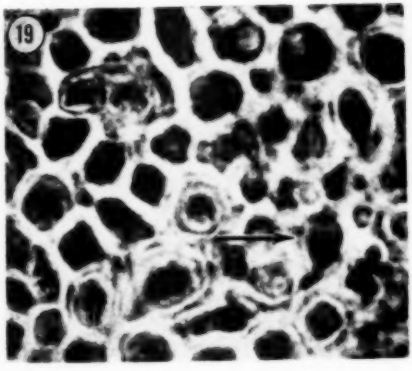
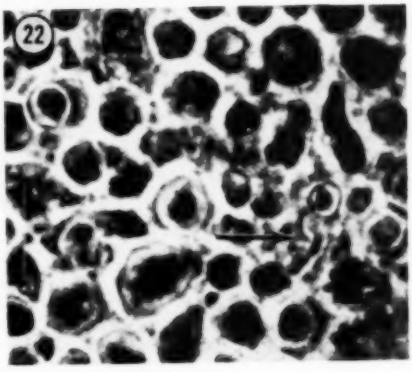




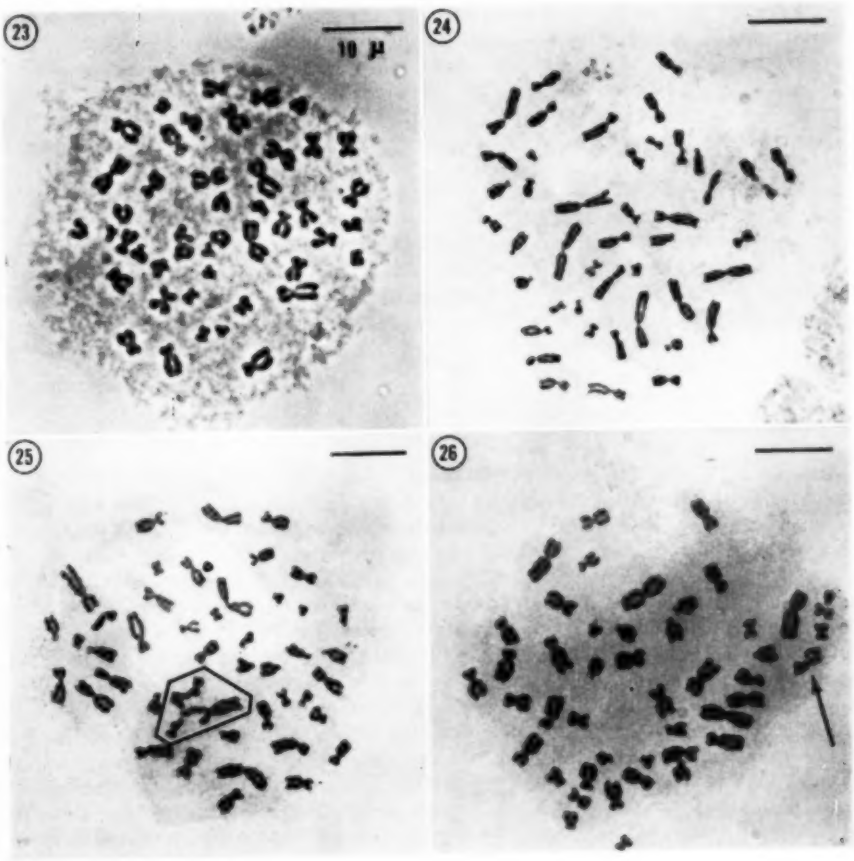








Richmond, Ohnuki, Awa, Pomerat.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Cell movement can be attributed to this active pulsatile movement of cytoplasm, since elements showing this activity are seen to move around the field. Progression appears to be haphazard, the myeloma cell showing slight darting movements in all directions, leading to final displacement from the original site. From Thiéry's description (1960) the rat plasma cell movement is a slower smoother process, characterized by cytoplasmic extension in one direction after which the rest of the cell becomes displaced along the same axis. Our own observations on the rat plasma cell confirm this impression. (3) Occasionally, myeloma cells show a single extended protrusion of cytoplasm, which develops from an area of rapid membrane undulation (Fig. 4, 5). This process remains stable for long periods, up to 15 minutes. At the tip of the process, however, there is very active undulation of the cell membrane. (4) Fine membranous extensions of cytoplasm may be seen on the periphery of the myeloma cell (Fig. 2). These thin protrusions extend and retract fairly rapidly, lasting about 20 seconds from start to finish, and appearing at many points on the circumference of the cell. This activity was seen by Thiéry in rat plasmablasts, and he has shown by electron microscopy that small droplets of fluid are imprisioned by these thin pseudopodia of cell membrane, constituting a micropinocytic process. Vacuoles have not been observed by phase contrast examination of myeloma cells but no doubt the vacuoles are too small to be observed by this technique.

Morphology of myeloma cells in tissue culture

Myeloma cells generally retained their characteristic morphology for as long as 6 weeks *in vitro*, which was the limit of the experiments. During the first three weeks, their numbers tended to be static and only an occasional mitotic figure was identified as certainly occurring in a plasma cell. In 1500 feet of cinematographic film, a single mitosis was seen in a typical myeloma cell (Fig. 10, 11, 12). Despite this, examination of cultures fixed and stained at intervals during the first two weeks showed an increase in the number of binucleate and multinucleate myeloma cells (Fig. 15), suggesting that growth, rather than maintenance, was being achieved in the culture chambers. In later weeks, however, the myeloma cells became less numerous, in contrast to an overwhelming fibroblastic outgrowth. This decrease in myeloma cells was probably due, in large part, to death of the cells but in part it was the result of transformation of occasional myeloma cells into a fibroblast-like element. Elongation of plasma cells to a spindle-shaped form was seen in many of the cultures, and photographic records were made using phase contrast microscopy. Examination of these small spindle cells in fixed and stained preparations (May-Grünwald-Giemsa) showed that the nucleus retained the characteristic chromatin network of the myeloma cell while the cytoplasm exhibited the typical pale juxta-nuclear zone and deep blue coloration elsewhere due to its high content of ribonucleic acid. At this stage it was simple to differentiate from the larger, pale fibroblast with its vesicular nucleus (Fig. 16).

Stained preparations of myeloma cells growing in culture often show a peculiar exaggeration of the normally eccentric position of the cell nucleus, so that the nucleus appears to lie partly outside the cytoplasm (Fig. 15). While this might have been considered a staining or fixation artefact, phase contrast photographs of living cells show an identical morphology (Fig. 3). We do not suggest that the

plasma membrane is not intact, but merely wish to draw attention to this characteristic appearance of the myeloma cell in culture.

Unlike epithelial and fibroblastic cells, myeloma cells do not flatten out well on the glass surface of the culture chamber. This propensity militated against good high-power observation and photography by phase-contrast optics. For instance, the endoplasmic reticulum was very difficult to demonstrate. Thiéry has obtained good phase-contrast photographs of the endoplasmic reticulum in the rat plasma cell, by examination in a thin preparation, and we have confirmed his results in similar preparations of rat plasma cells. In the case of human myeloma cells, we have dismantled Rose chamber cultures and placed the chamber coverslip on a glass slide with a little fluid between the two surfaces. Under these conditions, the myeloma cells remain active for 15 to 30 minutes, and oil-immersion photography of more flattened cells is rendered possible. The endoplasmic reticulum has been identified and recorded (Fig. 6) in the living myeloma cell, although only in cells which were degenerating and about to die.

Effect of treatment with NSC-1026 in vitro

Cultures of myeloma tumour and bone-marrow tissue were exposed to standard medium containing 0·2, 0·5, 0·75 and 1 per cent NSC-1026. Since these are relatively high concentrations, it should be stressed that the effective dosage of a drug *in vitro* may not run *pari passu* with the dosage in man (Painter, Pomerat and Ezell, 1949). Cinematographic records of cell activity were made on some of the preparations while others were treated for 1 to 3 days followed by fixation and staining. With 0·2 per cent NSC-1026, no visible change was detected in living or fixed material. Cultures exposed to 1·0 per cent NSC-1026 showed evidence of cell damage and death within one hour; most cells were killed by 12 hours treatment and all cells were dead at 30 hours. Continuous treatment with 0·5 and 0·75 per cent NSC-1026 over a period of 3 days led to death of about half the cells in the culture.

Using time-lapse cinematography, we were able to analyse the dynamic changes associated with toxicity at dose levels of 0·5 and 0·75 per cent. The most common change which indicated severe cell damage and early death was the cessation of membrane undulation. Degeneration and rounding up of the cell body occurred a few minutes after the cell membrane movement stopped. One peculiar feature, not necessarily presaging death, was a vigorous rocking movement in the myeloma nucleus. The nucleus would rock back and forth through an angle of 160–180°, taking an average of 16 minutes to complete each cycle. A small percentage of myeloma cells responded in a different and unusual manner: these cells became elongated, showing spasmodic contractions and elongations over a period of 4 to 10 hours (Fig. 17–22), associated with rapid intracellular movements of the nucleus along the long axis of the elongated cell. Eventually the cell would degenerate and become rounded up and granular in appearance. We had no way of ascertaining, from the ciné records, whether any particular maturation stage of the myeloma cell was responsible for the activity described.

Examination of stained preparations showed that the cells in the original explant, as well as in the outgrowth, were killed and subsequently lysed, following exposure to 1 per cent NSC-1026 (Fig. 13, 14). At lower levels of dosage we could not detect any specific action of NSC-1026—adventitial cells and myeloma cells appeared to be affected equally.

Chromosomal investigation

On examination of the buffy coat culture of Case 1 before treatment, 50 well-spread metaphase plates were available for study. Each of these cells had the diploid complement of 46 chromosomes which were of normal morphology. In preparations obtained after treatment of the patient with NSC-1026, a total of 221 well-spread metaphase plates were examined. The results are shown in Table I.

TABLE I.—*Analysis of Metaphase Plates in Buffy Coat Culture Following Treatment with NSC-1026*

Chromosome morphology	Number of cells
Normal . . .	215*
Abnormal karyotype	2
Chromosome breakage .	4†

* Four showed variation in chromosome number—47(2), 48(1), 50(1).
† Estimated chromosome number, 46. Two cells showed typical translocation.

Some variation in the chromosome number, similar to that detailed in the table, has been reported by other workers (Nowell and Hungerford 1960) in tissues grown *in vitro* and has been the common experience in our own laboratories (Ohnuki *et al.*, 1962). Similarly, we have observed chromatid breakage in control cultures of normal adult humans. Quite unusual, however, in our hands is the finding of 2 cells with abnormal karyotype (Fig. 23, 24, 27, 28) and 4 cells showing chromosome breakage and translocation (Fig. 25, 26). There can be little doubt that these changes represent an effect of *in vivo* treatment with NSC-1026. Indeed, chromosome breakage and translocation is a common event following treatment of growing cells with X-rays or radiomimetic drugs (Kaufmann, 1954; Lea, 1955; Biesele, 1958). It must be stressed that there is no way of knowing which circulating blood cells have undergone mitosis in buffy coat cultures of our patient. Monocytes and possibly lumphocytes are thought to be responsible for the usual growth in this type of culture. Smear examination of the blood in our patient showed the presence of occasional plasmablasts and more mature plasma cells, and therefore some presumably malignant cells may have contributed to the mitoses observed, in the samples taken before and after treatment. While this uncertainty remains, it is clear that buffy coat culture can help with the estimation of the efficacy of anti-tumour chemotherapy. We can judge whether the dose-level is sufficient to cause toxic changes in the patient's cells, as shown by induced chromosomal abnormalities. Recent reports of allied nature have shown that chromosomal damage can result from therapeutic doses of radio-iodine (Boyd, Buchanan and Lennox, 1961) and the therapeutic or diagnostic use of X-rays (Tough *et al.*, 1960; Stewart and Sanderson, 1961).

Chromosome studies of bone marrow tissue were successfully completed in pre-treatment samples from Case 1 and Case 3. Numerous myeloma cells were present in both specimens. In Case 1, 30 metaphase plates showed chromosomes of normal configuration, 28 cells having 46 chromosomes, 1 with 47, and 1 with 92. The findings were essentially similar in 30 metaphase plates from Case 3, namely 29 cells with 46 chromosomes and 1 cell with 92 chromosomes, all of normal morphology. Post-treatment samples did not grow successfully.

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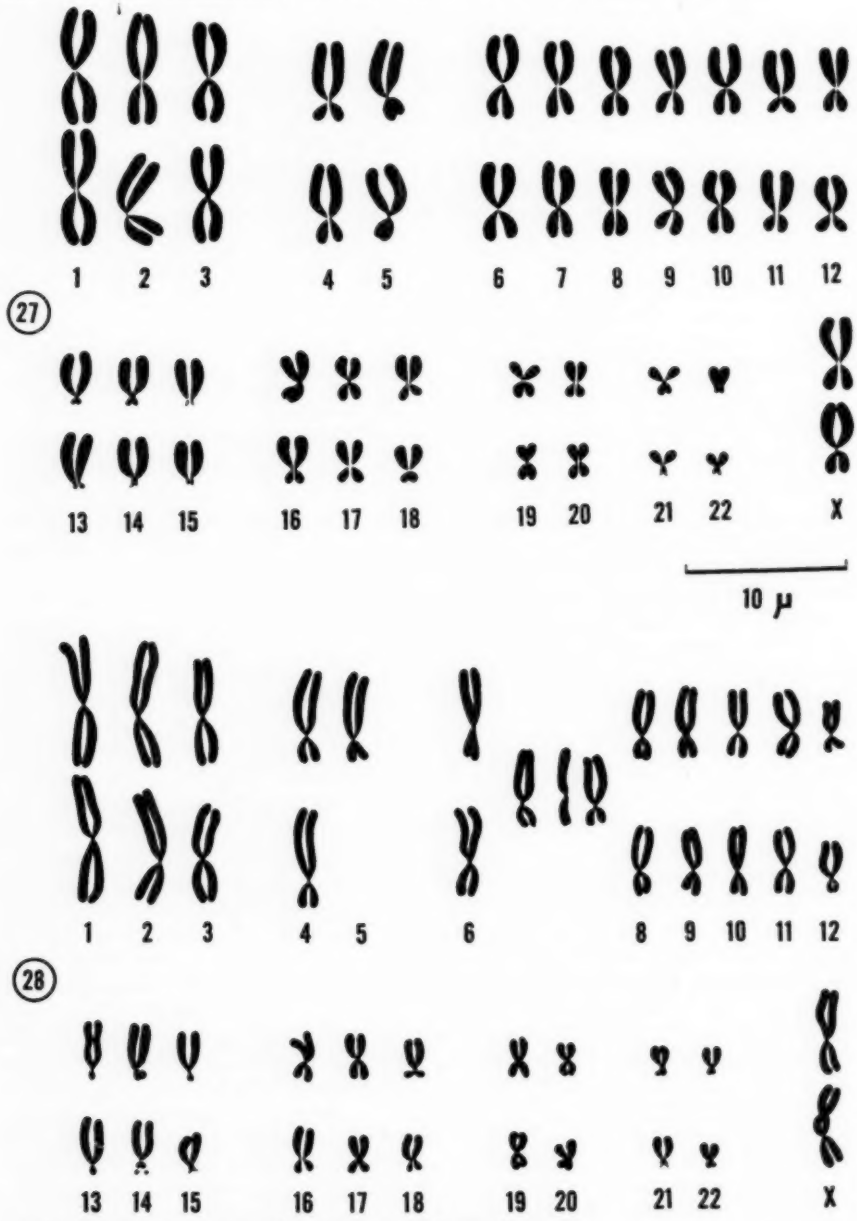

FIG. 27.—Normal karyotype from buffy coat culture of Case 1.
FIG. 28.—Abnormal karyotype following treatment with NSC-1026 (Idiogram of same metaphase plate depicted in Fig. 24).

SUMMARY AND CONCLUSIONS

Cultures of myeloma cells, from three cases of multiple myeloma, were grown *in vitro* and their dynamic behaviour was recorded by time-lapse cinematography, using phase-contrast optics. Myeloma cells show characteristic movements in the cell membrane, (1) rapid undulation, (2) pulsating pseudopodal extensions of rhythmic nature, (3) a pseudopodal extension of cell cytoplasm similar to (2) in shape but relatively static, (4) short fine membranous protrusions which extend and retract rapidly. These activities are similar to those demonstrated by Thiéry (1960) in the normal rat plasma cell, and he has shown by electron microscopy that the last type of membrane movement constitutes a process of micropinocytosis. We can conclude that the dynamic behaviour of the myeloma cell is essentially similar to the plasma cell.

Morphological changes observed in tissue culture indicate that some myeloma cells can transform into elongated spindle cells. The abundant endoplasmic reticulum which is so characteristic of the plasma cell and the myeloma cell observed with electron microscopy (Braunsteiner *et al.*, 1957) has not been seen by us in healthy myeloma cells using phase contrast optics, but we have been able to demonstrate endoplasmic reticulum in the degenerating cell, when vacuolization of the endoplasmic network is in progress.

Treatment of myeloma cells *in vitro* with 0·2 to 1 per cent 1-amino-cyclopentanecarboxylic acid (NSC-1026) has demonstrated a peculiar reaction to chemotherapy. The myeloma cells show spasmodic elongation and contraction, while the nucleus repeatedly traverses from one end to the other of the elongated cell. Finally, the damaged cell becomes rounded up and shows irreversible injury. This reaction to chemotherapy appears to be characteristic for the plasma cell and we have not observed such a change in previous time-lapse studies employing cancer chemotherapeutic agents.

Buffy coat cultures of the peripheral blood failed to demonstrate any specific chromosome " marker " in multiple myeloma. However, examination of buffy coat cultures after *in vivo* treatment with NSC-1026, in one patient showed the formation of abnormal karyotypes and chromosomal translocations, contrasting with the normal appearances before treatment. We suggest that this technique of buffy coat culture before and after therapy could give valuable information regarding the efficacy and appropriate dosage level of newly elaborated chemotherapeutic agents, when these are being assessed in human cancer.

We are indebted to Dr. W. Benefiel and Dr. H. Moorman, of the Pasadena Tumor Institute, for supplying material from myeloma patients. Mr. Charles Raiborn and Mr. George Lefeber were responsible for the tissue culture and photographic techniques involved in his study.

This investigation has been aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research and in part by the U.S. Army Medical Research and Development Command, Department of the Army, under Research Grant No. DA–MEDDH–61–12.

REFERENCES

Benefiel, W. W., Helsper, J. T. and Sharp, G. S.—(1960) *Cancer Chemotherapy Rep.*, **9**, 21.

UMI

BIESELE, J. J.—(1958) In 'Mitotic Poisons and the Cancer Problem'. New York (Elsevier Press Inc.).
BOTTURA, C., FERRARI, I. AND VEIGA, A. A.—(1961) *Lancet*, i, 1170.
BOYD, E., BUCHANAN, W. W. AND LENNOX, B.—(1961) *Ibid.*, i, 977.
BRAUNSTEINER, H., FELLINGER, K. AND PAKESCH, F.—(1957) *Blood*, **12**, 278.
DALTON, A. J., POTTER, M. AND MERWIN, R. M.—(1961) *J. nat. Cancer Inst.*, **26**, 1221.
DENMAN, A. M. AND WARD, H. W. C.—(1960) *Brit. med. J.*, i, 482.
HAYES, D. M., SPURR, C. L. AND HINES, J. D.—(1961) *Proc. Amer. Ass. Cancer Res.*, **3**, 233 (Abstract).
HUNGERFORD, D. A., DONNELLY, A. J., NOWELL, P. C. AND BECK, S.—(1959) *Amer. J. hum. Genet.*, **11**, 215.
KAUFMANN, B. P.—(1954) In 'Radiation Biology'. Edited by A. Hollaender, **1**, Part II. New York (McGraw-Hill Book Co.), p. 627.
KAY, H. E., LEDLIE, E. M. AND SBRESNI, R. C.—(1959) *Brit. J. Radiol.*, **32**, 791.
LEA, D. E.—(1955) In 'Action of Radiations on Living Cells' (2nd Ed.). London (Cambridge University Press).
NATHAN, D., FAHEY, J. L. AND POTTER, M.—(1958) *J. exp. Med.*, **108**, 121.
NOWELL, P. C.—(1960) *Cancer Res.*, **20**, 264.
Idem AND HUNGERFORD, D. A.—(1960) *J. nat. Cancer Inst.*, **25**, 85.
OHNUKI, Y., AWA, A. AND POMERAT, C. M.—(1962) *Ann. N.Y. Acad. Sci.*, In Press.
PAINTER, J. T., POMERAT, C. M. AND EZELL, D.—(1949) *Tex. Rep. Biol. Med.*, **7**, 417.
ROSE, G. C., POMERAT, C. M., SHINDLER, T. O. AND TRUNNELL, J. B.—(1958) *J. biophys. biochem. Cytol.*, **4**, 761.
STEWART, J. S. AND SANDERSON, A. R.—(1961) *Lancet*, i, 978.
THIÉRY, J. P.—(1960) In 'Ciba Foundation Symposium on Cellular Aspects of Immunity'. Edited by G. Wolstenholme and C. O'Connor. Boston (Little, Brown and Co.), p. 59.
TOUGH, I. M., BUCKTON, K. E., BAIKIE, A. G. AND COURT-BROWN, W. M.—(1960) *Lancet*, ii, 849.
TRUAX, W. E., BRAY, J. AND PERRY, J. E.—(1961) *J. Lab. clin. Med.*, **57**, 54.
VASQUEZ, J. J.—(1958) *Ibid.*, **51**, 271.

SURVEY OF PAPERS

KORPELA and MAGNUS have analysed the available data on lung cancer morbidity and mortality from Finland and Norway, official vital statistics from these two countries having revealed a large excess in the latter (p. 393).

CARMICHAEL and SILVERSTONE have calculated the age specific incidence rates of skin cancer in four coastal cities in Queensland. They discuss their findings and suggest, on the assumption that ultraviolet radiation is the causative factor, and from the observation that the curves are parallel for each of the four cities, that the only difference in response to the stimulus by the four populations is one of frequency of incidence (p. 409).

CARMICHAEL shows that, in Queensland, there is a tendency for both basal and squamous cell cancer to occur in the absence of hyperkeratosis, and that the converse also holds. Solar keratosis does not appear to be an important premalignant lesion (p. 425).

WHITWELL reviews ten years experience of bronchial biopsy histology, discussing the benign tumours found, and assessing the degree of accuracy of the histological diagnosis of lung cancer, and the degree of consistency found in reporting cell types of lung cancer (p. 429).

WHITWELL analyses the histopathology of over 1900 cases of lung cancer occurring in Liverpool in a decade in three series in which tissues were obtained from bronchial biopsy, operation specimens and post mortem specimens. Different cell-type frequencies found in these series were explainable by the biological properties of each cell-type, and by external factors influencing the selection of cases. None of these types of series reflects the true cell-type incidence of lung cancer in a population (p. 440).

JOLLES surveys 117 cases of bronchial carcinoma treated by X-ray sieve therapy. He claims that the sieve technique gives better results in comparison with conventional methods of treatment for this disease (p. 460).

BRIDGES, HAYES and NELSON record the results of treatment of 25 cases of chronic granulocyte leukaemia by busulphan. Of 16 patients given this drug as initial therapy, 14 made satisfactory responses (p. 468).

ABUL-FADL and KHALAFALLAH present a preliminary report on the urinary secretion of certain tryptophan metabolites in 220 cases. They find an increase of 3-hydroxyanthranalic acid to twice the normal levels in cases of bilharzial affections, and to four times in bilharzial cancers of the bladder (p. 479).

GRUBB and CRABBE compare the results of acridine orange fluorescence microscopy of a series of cytological specimens with the Papanicolaou method, to the advantage of the former technique (p. 483).

GADEKAR and SAHASRABUDHE show that the radiation sensitivity of tumours is augmented by pretreatment with "dicetol", a substance which is non-toxic in therapeutic doses (p. 489).

BOYLAND, STAUNTON and WILLIAMS perfused the limbs of dogs with Chlorambucil and Melphalan and determined the uptake of the drugs. Chlorambucil was rapidly taken up by tissues from the circulating blood. Concentrations of Chlorambucil below 200 μl./ml. did not cause any damage to the limb when perfused at 37·5°, but some damage resulted if this concentration was administered at higher temperatures (p. 498).

WIGHT describes a cholangiocellular carcinoma occurring in a domestic fowl and compares the structure, incidence and aetiological factors associated with this type of tumour with similar neoplasms of mammals (p. 511).

ROE, ROWSON and SALAMAN find that a single dose of 30μg. 9,10-dimethyl-1, 2-benzanthracene given subcutaneously to newborn mice of two strains gives rise to tumours of many sites. Their results suggest that newborn mice would be useful in screening substances for carcinogenic action. Certain immunological concepts if carcinogenesis are criticised (p. 515).

GLEDHILL finds that intraperitoneal inoculation of weanling mice with leukaemia agent a few days before inoculation with mouse hepatitis virus greatly increases the pathogenicity of the latter (p. 531).

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Adams has measured body and liver weights, liver total N, liver weight/body weight ratio, total, granule, and EPC liver catalase activities, and intracellular catalase distribution in mice bearing sarcoma 37, pregnant mice, and mice kept on diets deficient in protein and/or riboflavin with or without treatment with cortisone. He draws various conclusions concerning the effect of tumours on their hosts (p. 539).

Mundy and Williams report on the mammary tumour incidence in a colony of BR6 mice and on the development of tumour-free sublines (p. 561).

Marchant reports the induction by methylcholanthrene of breast tumours in C57Bl mice. While virgins are resistant, the hormonal conditions of pseudopregnancy and breeding favour development of tumours. Lactation delays, but does not prevent, their appearance (p. 568).

Pullinger reports an increase in the incidence of mammary carcinoma from 2 per cent in intact C_3Hf virgin female mice to 25 and 26 per cent respectively of those ovariectomised and given oestrone alone or a mixture of oestrone, oestriol and oestradiol weekly for 60 weeks. The incidence of adenomas is also greatly increased (p. 574).

Pikovski and Witz report the presence of a specific tissue antigen of globulin nature in the mammary glands of RIII mice which is lacking in the mammary tumours of these mice. They have also shown a considerably increased concentration of an immunologically distinct antigen in spontaneous and transplanted mammary tumours of the same strain (p. 584)

Richmond shows that the toxic effects of iron-dextran on cells *in vitro* are counteracted by traces of cobalt, suggesting that an oxidative reaction is responsible for iron toxicity. Iron-dextran produces cytological changes similar to those seen after X-irradiation (p. 594).

Cater finds that rats injected subcutaneously with the sulphonated polygalactose carrageenin develop sarcomata. One dose of the carcinogenic acylethyleneimines into the carrageenin-induced granuloma does not increase tumour incidence significantly. Carrageenin also causes fibrous replacement of mammary gland, hyaline thickening of capillary walls and prolonged loss of hair (p. 607).

Head and Laird report studies of the histological appearances of hepatoma grafts each week from the time of grafting till a tumour becomes palpable. They find that not all grafts grow in early transplant generations, although hosts are genetically comparable and the tumours are potentially malignant (p. 615).

Hou and Rees have followed the histological and biochemical events occurring in acetylaminofluorene hepatocarcinogenesis in rats. They find that an increase in nuclear material and a fall in the cytoplasmic fraction and in the number of mitochondria precedes the development of hepatocarcinoma (p. 624).

Neish and Rylett report the presence of a substance, apparently a thiol peptide closely related to glutathione, in the livers of hepatocarcinogen treated rats. They discuss its occurrence and nature (p. 630).

Siegel, Graig, Crystal and Siegel have studied the distribution in man of administered ^{65}Zn. By determining the concentrations of the radioisotope in various organs, especially the prostate, estimates of total organ uptake were made. Radioautography was employed to investigate the cellular localization of the tag within the prostate and, also, by bone marrow elements (p. 647).

Hartveit reports that abrogation of the natural immunity of mice to Ehrlich's ascites carcinoma, by means of trypan blue and cortisone, reduces the blood content of the tumour, indicating that the haemorrhagic reaction is an expression of their immunological response to the tumour (p. 665).

Calcutt has summarised the evidence for the hypothesis that a rise in target tissue sulphydryl levels is an essential feature of tumour induction. Other evidence in the literature has also been considered and found consistent with this hypothesis (p. 673).

Calcutt has found the feeding of ethionine to rats to cause prolonged elevations of liver –SH levels, but to have little effect on kidney –SH levels. Histological changes in the liver have also been examined (p. 683).

Hnilica and Holoubek have studied the action of histone, deoxyribonucleoprotein and deoxyribonucleic acid prepared from leukaemic rat liver and spleen and from calf thymus in newborn rats. None of the injected substances caused malignant changes (p. 687).

Richmond, Ohnuki, Awa and Pomerat have studied in tissue culture the dynamic morphology of human myeloma cells and find their behaviour is the same as normal rat plasma cells. Characteristic toxic reactions are seen in myeloma cells after *in vitro* treatment with 1-aminocyclopentanecarboxylic acid. Similar chemotherapy *in vivo* leads to chromosomal abnormalities in buffy coat culture of peripheral blood (p. 692).

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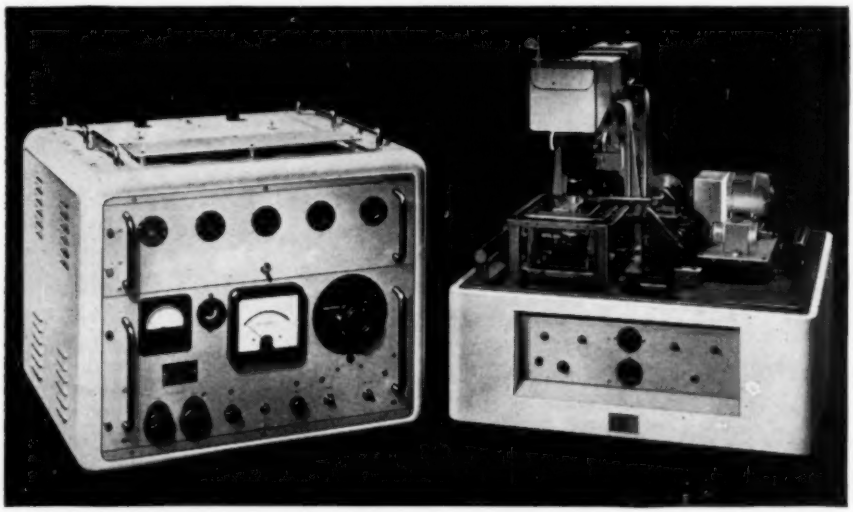

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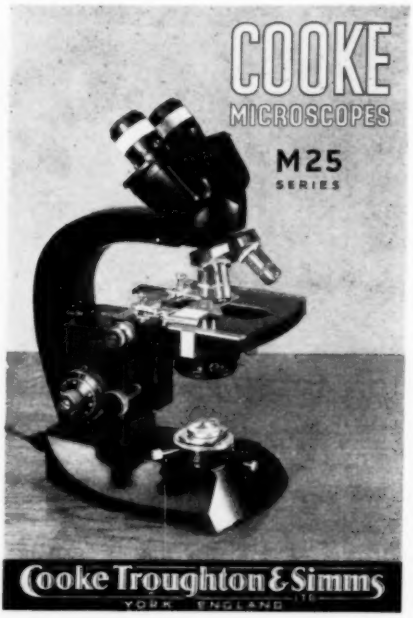

British Empire Cancer Campaign

A SURVEY OF CANCER IN LONDON

REPORT OF THE CLINICAL CANCER RESEARCH COMMITTEE

by W. L. HARNETT,
C.I.E., M.D., F.R.C.S,
Medical Secretary to the Committee.
With a FOREWORD by the
RT. HON. LORD HORDER,
G.C.V.O., M.D., F.R.C.P.,
and an INTRODUCTION by
SIR HENEAGE OGILVIE,
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